respectively, of the controls. Combination treatment with tiazofurin (500 mg/kg/d) and taxol (12.5 mg/kg/d) reduced the tumor size to 51%, indicating synergism. Synergism was also observed when tiazofurin (700 mg/kg/d) was given followed 8 h later by taxol (12.5 mg/kg/d) which reduced the tumor size to 34% of the control. These results indicating synergism of taxol with tiazofurin should be of interest in the treatment of relapsed inoperable ovarian carcinoma. (Supported in part by grant #IRG-161J to F.S. from the American Cancer Society.)

#2563 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 13

Intermittent exposure to paclitaxel resulting in high levels of non-MDR paclitaxel resistance in the human ovarian carcinoma cell line SKOV3. Rischin, D., Lee, G., Cursisinghe, A., Woodcock, D. *Division of Haematology and Medical Oncology and Treatswithick Research Laboratories, Peter MacCallum Cancer Institute, Melbourne, Australia.*

Two cell lines, SKTAX3b and SKTAX6a, have been established by exposing the human ovarian carcinoma cell line SKOV3 to intermittent, step-wise increasing concentrations of paclitaxel. SKTAX3b and SKTAX6a are 30 and 300 fold resistant to paclitaxel and 6 and 77 fold resistant to docetaxel respectively. Both cell lines exhibit low levels of resistance to vinca alkaloids but no significant resistance to colchicine, etoposide or doxorubicin. There is collateral sensitivity to cisplatin. There is no overexpression of MDR1 on Northern analysis or of P-glycoprotein on flow cytometry using MRK-16. The percentage of cells arrested at G_2/M following exposure to 250nM paclitaxel is much lower in the resistant cell lines than in SKOV3. β and α tubulin levels on Western blot analysis are decreased in SKTAX3b but not in SKTAX6a. Paclitaxel accumulation and expression of tubulin isotypes are currently being investigated.

#2564 Sunday, April 21, 1996, 1:00–5:00, Room 20

Potentiation of the cytotoxicity of anticancer agents by novel poly(ADP-ribose)-polymerase (PARP) inhibitors. Bowman, K.J.,[a] Calvert, A.H.,[a] Curtin, N.J.,[a] Golding, B.T.,[b] Griffin, R.J.,[b] Newell, D.R.,[a] Srinivasan, S.[b] and White A.[b]. *Cancer Research Unit[a] and Department of Chemistry[b], University of Newcastle upon Tyne, UK.*

The nucleus enzyme poly(ADP-ribose)polymerase (PARP; EC 2.4.2.30) is activated by DNA strand breaks to form ADP-ribose polymers on nuclear acceptor proteins, which causes relaxation of chromatin in the region of the strand break and facilitates DNA repair. Thus, PARP inhibition has the potential to enhance the cytotoxicity of DNA damaging anticancer therapy. Novel series of quinazolin-4-[3*H*]-one and benzimidazole-4-carboxamide PARP inhibitors have recently been developed with IC_{50} values for PARP inhibition ≤1 μM. Clonogenic assays using L1210 cells were performed to investigate the potentiation of cytotoxic agents by these novel inhibitors. The quinazolinone PARP inhibitor, NU1025 (IC_{50} = 400 nM), potentiated MTIC, the active methylating species derived from temozolomide and DTIC (enhancement factor at 10% cell survival (EF_{10}) of 4.1). Potentiation of the topoisomerase I inhibitor, camptothecin, (EF_{10} = 2.6), γ-irradiation (EF_{10} = 1.4) and bleomycin (EF_{10} = 1.4) was also observed. However, there was no potentiation of the topoisomerase II inhibitors, VP16 and doxorubicin, nor of the thymidylate synthase inhibitors, CB3717 and AG337. NU1064, a benzimidazole carboxamide PARP inhibitor (IC_{50} = 1 μM), significantly enhanced temozolomide cytotoxicity in a concentration-dependent manner. Studies are underway to identify the most suitable drug combinations for pre-clinical *in-vivo* evaluation.

#2565 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 13

Drug delivery systems: water soluble taxol-2'-polyethylene glycol ester prodrugs-design and in vivo effectiveness. Pendri, A., Greenwald, R., Gilbert, C., and Conover, C. *Enzon Inc. 20 Kingsbridge Road, Piscataway, NJ. 08854.*

PEG derivatives of molecular weight 5–40 kDa were condensed with paclitaxel to provide water soluble (120–660 mg/mL) 2'-taxol PEG-esters (alpha alkoxyesters). Rates of hydrolysis in buffer and rat plasma were determined for all compounds synthesized, and for a given linker were identical regardless of molecular weight. These compounds were shown to function as prodrugs (transport forms). The IC_{50} values of the prodrugs were, within experimental error, similar to native paclitaxel. However, in vivo experiments clearly established that in order for these prodrugs to behave in a predictable fashion, the molecular weight of PEG must be of such magnitude so as to maintain a $t_{1/2}$ circulation > $t_{1/2}$ hydrolysis. Only in the cases of PEG of molecular weight > 30kDa could equivalent potency of the prodrug and paclitaxel be demonstrated.

#2566 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 13

Polymer delivery of carmustine, 4-hydroperoxy-cyclophosphamide and taxol in the monkey brain. Pung, L.K., Ewend, M., Sills, A., Sipos, E., Thompson, R., Brem, H., and Saltzman, W.M. *Johns Hopkins University, MD 21218.*

The distribution of carmustine (BCNU), 4-hydroperoxy-cyclo-phosphamide (4-HC) and taxol following release from polymer implants in the monkey brain was studied. These agents were radiolabeled, and encapsulated into copolymers of carboxyphenoxypropane and sebacic acid (20:80 ratio). The drug-loaded polymer implants (20% loaded) were then inserted intracerebrally in monkeys. Average concentrations of agents over coronal sections of the brains were obtained by thin-layer chromatography and scintillation counting. Three days after polymer implantation, at ~1 mm away from the polymer, the concentrations were ~300μM for BCNU, ~20μM for 4-HC and ~30μM for taxol. One week following polymer implantation, all 3 agents were found to be present both ipsilaterally (~600nM, ~80nM and ~10nM for BCNU, 4-HC and taxol, respectively) and contralaterally (~300nM, ~70nM and ~6nM for BCNU, 4-HC and taxol, respectively). These findings indicate that polymer delivery of BCNU, 4-HC and taxol resulted in high concentrations near the implantation site and low, but significant, concentrations at distant sites in the primate brain.

#2567 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 15

Determination of urinary 6-beta-hydroxycortisol and cortisol ratio by capillary electrophoresis. Lin, S., Fleisher, M., Seidman, A., Spriggs, D., and Tong, W.P. *Memorial Sloan-Kettering Cancer Center, New York NY 10021.*

Cytochrome P450 3A enzymes metabolize many drugs such as etoposide, tamoxifen, taxol, vinblastine, nifedipine and terfenadine. Inhibitors like cyclosporin, ketoconazole and grapefruit juice can alter the pharmacokinetics of such drugs. Due to the broad interindividual variability and possible ethnic polymorphism, in a study with taxol, we examined the relationship of taxol pharmacokinetics and individual 3A4 pretreatment evaluation. Urinary 6-beta-hydroxycortisol/cortisol ratio has been used as endogenous marker to measure human 3A activity. Since this procedure is non-invasive and does not require the use of any test compound, we choose this method to correlate with the taxol pharmacokinetic parameters. After solid phase extraction of urine samples, capillary electrophoresis assay using a borate buffer with SDS, deoxycholate and methanol as electrolyte, achieved separation of both compounds in 10 minutes when compared to the HPLC with gradient elution procedure which required 50 minutes.

#2568 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 13

Activity and schedule dependent interactions of paclitaxel, etoposide and ifosfamide in cisplatin-sensitive and cisplatin-refractory human ovarian carcinoma cell lines. Klaassen U., Harstrick A, Schleucher N, Vanhoefer U, Schröder J, Wilke H, Seeber S. *Department of Internal Medicine (Cancer Research), West German Cancer Center, University of Essen, Hufelandstr. 55, 45122 Essen, Germany*

Paclitaxel has demonstrated broad clinical activity in a variety of malignancies both alone and in combination with other chemotherapeutic agents. The in vitro cytotoxicity of a 2h exposure to paclitaxel, hydroperoxy-ifosfamide and etoposide alone, in combination and in sequence, was evaluated against established cisplatin-sensitive A 2780 WT, TR170) and cisplatin-refractory (A 2780 CP2, TR 170/731) human ovarian carcinoma cell lines using isobologram analysis. The combinations of either paclitaxel/ifosfamide or paclitaxel/etoposide were found to be additive or synergistic when the drugs were given simultaneously or when paclitaxel was given 24 h prior to ifosfamide or etoposide, respectively. However, when etoposide or ifosfamide were given prior to paclitaxel, antagonistic interactions were observed. With regard to etoposide this antagonism was evident for up to 24 h. In concordance to our data with the schedule dependent interactions of paclitaxel and cisplatin in human gastric and ovarian carcinoma cell lines, these data demonstrate that the interactions of paclitaxel, etoposide and ifosfamide are also highly schedule dependent and applications of etoposide or ifosfamide prior to paclitaxel may result in pronounced antagonism. These findings could have implications for the design of further clinical protocols.

#2569 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 13

Synthesis and evaluation of PEG-paclitaxel conjugate as a water-soluble paclitaxel prodrug. Li, C., Yu, D-F., Inoue, T., Yang, D. J., Milas, L., Hunter, L. R., Wallace, S. *Univ. Texas M.D. Anderson Cancer Ctr., Houston, TX*

Because of its poor solubility in water, paclitaxel (Taxol) is formulated with Cremophor and alcohol. The vehicle has several toxic effects. We synthesized a water-soluble polyethylene glycol (PEG)-paclitaxel conjugate from C-2' succinyl paclitaxel and methoxypolyethylene glycol amine by an EEDQ-mediated coupling reaction. This anionic paclitaxel prodrug was highly water soluble (>20 mg/ml). The release of paclitaxel in phosphate buffered solution (pH 7.4) from PEG-paclitaxel conjugate was biphasic with $t_{1/2\alpha}$ of 42 min and $t_{1/2\beta}$ of 61 hrs. PEG-paclitaxel conjugate inhibited growth of B16 melanoma cells to an extent similar to that of paclitaxel. In MCA-4 mammary tumor-bearing mice, a single dose of PEG-paclitaxel (40 mg equiv. paclitaxel/kg body weight) delayed tumor growth. The average number of days tumors required to reach 12 mm in diameter increased from 6.5 days for control animals to 8.5 days for PEG-paclitaxel treated animals and 9.4 days for paclitaxel treated animals. The results indicate that water-soluble polymer can be used as solubilizing agent for paclitaxel and that the polymer-paclitaxel conjugate preserves its in vitro cytotoxicity and to a lesser degree, its in vivo antitumor efficacy.

#2570 Wednesday, April 24, 1996, 8:00–12:00, Poster Section 13

Cytotoxic and antitumor activity of water-soluble paclitaxel prodrug. Li, C., Yu, D-F., Inoue, T., Yang, D.J., Milas, L., Hunter, N.R., Wallace, S. *Univ. Texas M.D. Anderson Cancer Ctr., Houston, TX 77030, USA.*

Because of its poor water solubility, paclitaxel is clinically formulated with Cremophor and alcohol. This vehicle is biologically active with toxic effects. We synthesized a DTPA-paclitaxel conjugate with improved aqueous solubility (>20 mg/ml) and

ubiquitinated following alkylation. The mutant C145A, which can not react with BG, was not immunoprecipitated. These data support the hypothesis that AGT, following alkylation at the active site is degraded by the ubiquitin/proteasomal pathway.

#2657 7-Hydroxystaurosporine (UCN-01) inhibits nucleotide excision repair (NER): Attenuation of ERCC1-XPA interaction. Jiang, H., Li, L., and Yang, L.-Y. *Lab. Med., UT M. D. Anderson Cancer Center, Houston, TX 77030.*

UCN-01, a specific PKC inhibitor, has recently been shown to act as a cell-cycle checkpoint regulator that abrogates S and G2 arrest induced by DNA-damaging agents. Since DNA damage-induced G2 arrest is believed to promote DNA repair before cells enter mitosis, UCN-01's abrogation of G2 arrest may imply that UCN-01 inhibits DNA repair. We determined whether and how UCN-01 inhibits NER of cisplatin-induced DNA adducts. Addition of UCN-01 (50 nM, 6 h) to cisplatin-treated A549 human lung cancer cells resulted in a 40% increase of platination, whereas the intracellular accumulation of platinum remained unaffected. In an *in vitro* repair assay using whole-cell extracts and a cisplatin-modified plasmid as the substrate, the extracts from UCN-01-treated cells showed a marked reduction in NER capacity compared to their untreated counterparts; the effect was UCN-01 dose-dependent (IC_{50}=125 nM). Although UCN-01 increased the expression of XPA and ERCC1repair protein, it reduced the translocation of ERCC1 to the detergent-insoluble, DNA-bound fraction; the ratio of ERCC1 to XPA in the insoluble fraction decreased as the concentration of UCN-01 increased. Incubation of immobilized MBP-XPA fusion protein with lysates from UCN-01-treated cells revealed that UCN-01 (250 nM) reduced ERCC1 binding to XPA by 64%, as measured by western blotting with an anti-ERCC1. These results show that UCN-01 inhibits NER and that the inhibition may result from UCN-01-mediated attenuation of the ERCC1-XPA interaction.

#2658 Phosphorylation of the DNA repair protein, O^6-alkylguanine DNA alkyltransferase (AGAT or MGMT) in human brain tumor cells. Srivenugopal, K.S., Shou, J., and Ali-Osman, F. *UT M. D. Anderson Cancer Center, Houston, TX 77030.*

Inactivation of AGAT to improve the efficacy of chloroethylating and methylating agents is a current clinical strategy with much promise. However, the regulation of AGAT function by post-translational modifications and their possible exploitation to improve chemotherapy has not been explored. In this study, we examined protein phosphorylation as a regulatory mechanism for AGAT in the human medulloblastoma cell line, UW228. Cell extracts incubated with [γ-^{32}P]-ATP showed Mg^{++} ion dependent phosphorylation of the endogenous AGAT. Exposure of UW228 cells to ^{32}P-inorganic phosphate (10 μCi/ml) followed by immunoprecipitation (ip) showed the existence of AGAT as a phosphoprotein under physiological conditions. Both tyrosine and serine phosphorylations were identified by a combination of ip and Western analysis. Dephosphorylation of cell extracts using alkaline phosphatase resulted in a significant loss of AGAT activity. Treatment of the recombinant AGAT protein with purified protein kinases led to increased AGAT activity. These data provide the first evidence for AGAT phosphorylation and suggest reversible phosphorylation as a novel mechanism to control the activity levels of the DNA repair protein [supported by R29CA74321 grant].

#2659 Potentiation of temozolomide and topotecan growth inhibition and cytotoxicity by poly(ADP-ribose) polymerase (PARP) inhibitors in a panel of human cancer cell lines. [1]Delaney, C.A., [1]Wang, L.-Z., [1]Kyle, S., [2]Srinivasan, S., [2]White, A.W., [1]Curtin, N.J., [1]Calvert, A.H., [1]Durkacz, B.W., and [1]Newell, D.R. *[1]Cancer Research Unit, [2]Department of Chemistry, University of Newcastle-upon-Tyne, NE2 4HH, UK.*

Inhibition of poly(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in the repair of DNA strand breaks, can potentiate the cytotoxicity of DNA damaging agents. We have investigated the ability of 2 classes of PARP inhibitors:- a quinazolinone (NU1025, PARP inhibition KI 50 nM) and a benzimidazole (NU1085, PARP inhibition KI 6 nM) to potentiate the *in vitro* activity of the monofunctional alkylating agent temozolomide (TM) and the topoisomerase I inhibitor topotecan (TPT). A panel of human tumour cell lines:- HT29, LoVo, LS174T (colon), MCF-7, T47D, MDA-231 (breast), SKOV3, A2780, OAW-42 (ovarian) and A549, OCRL23, H522 (lung) were treated with increasing concentrations of either TM or TPT together with NU1085 (10 μM) and NU1025 (50 and 200 μM). Potentiation of growth inhibition (72 h continuous exposure, SRB assay) varied between cell lines, and was 1.5-4 fold for TM and 2.5-6 fold for TPT (n = 2-3 separate experiments). Clonogenic survival assays, undertaken in 5 of the cell lines, confirmed potentiation of TM and TPT toxicity. NU1085 (10 μM) prevented poly(ADP-ribose) (polymer) formation in intact A549 cells as demonstrated by immunofluorescence.

#2660 Mdm2 sensitizes breast cancer cells to cisplatin or carboplatin. Smith, M.L. *Indiana University Cancer Center, Dept. of Microbiology/Immunology, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202.*

Cisplatin and other platinum compounds produce DNA lesions that, akin to those produced by UV-light, are repaired by the nucleotide excision repair (NER) pathway. We and a number of others have shown that NER is partially defective in cells lacking functional p53, and that cells lacking functional p53 are (in some cell backgrounds) preferentially sensitized to DNA-damaging agents e.g. UV radiation or cisplatin. Because Mdm2 appears to regulate p53 in normal cells, and is oncogenic in some cancers including breast cancers, we tested whether suppression of p53 by Mdm2 would recapitulate the effect of p53 mutation on (on NER and platinum sensitivity). MCF7 breast carcinoma cells (wild-type p53 stably transfected with Mdm2 are sensitized to cisplatin or carboplatin. This is due in part to suppression of NER concomitant with suppression of p53-downstream effectors e.g. Gadd45. P53-independent mechanisms, possibly related to cell cycle control, may also contribute to platinum sensitivity. Effects of Mdm2 are being studied in mutant-p53 breast cancer lines.

#2661 Increased poly (ADP-ribose) polymerase expression, activity, and sensitivity to ionizing radiation in DU145 cells exposed to human recombinant interferon alpha-2a. Damowski, J.W., Goulette, F.A., Whartenby, K.A., and Calabresi, P. *Dept. of Med., Brown University and Rhode Island Hospital, Providence, RI, 02903.*

We reported that exposing DU145 human prostate tumor cells to human recombinant interferon alpha-2a (IFN) for 24h increased their sensitivity to ionizing radiation (IR) in an IFN concentration-dependent manner. Now we report that effect correlates with an increase in poly (ADP-ribose) polymerase (PARP) expression and intracellular NAD^+ content, both of which are IFN-concentration dependent. IFN exposure also increases PARP activity. Exposing DU145 cells to 10Gy of IR reduces intracellular NAD^+ pools by ~20% which then return to normal within 90min. In IFN pre-exposed (24h) cells, 10Gy reduces NAD^+ by 50% and this pool does not return to normal until 4-5h later. To further evaluate the relationship between IFN exposure, sensitivity to IR, and changes in PARP function/activity, 3-amino-benzamide (3AB), an inhibitor of PARP activity, was employed. A 24h exposure to 1.5mM 3AB (±IFN) does not affect sensitivity to IR in these cells. However, this exposure to 3AB does increase PARP expression to a degree similar to that observed after IFN (±3AB) exposure. Finally, a pre-exposure to 3AB (±IFN) prevents NAD^+ pool depletion following 10Gy of IR. These findings reveal that IFN exposure increases PARP expression and activity in this model. However, it is the increase in PARP activity which correlates with increased sensitivity to IR. The cellular and therapeutic consequences of these studies will be discussed (RIH and TJ Martell Foundation).

#2662 Relationship between unscheduled DNA synthesis and increased the expression of ribonucleotide reductase protein in chronic lymphocytic leukemia cells. Rodriguez, M. and Gandhi, V. *The University of Texas M. Anderson Cancer Center, Houston, TX 77030.*

Modifications in the regulation of enzymes may be required for repair of DNA damage caused by therapeutic agents. Enzymes such as ribonucleotide reductase (RNR), necessary for DNA replication, are found at low endogenous levels in quiescent cells. We hypothesized that in such cells initiation of DNA repair caused by alkylating agents such as cyclophosphamide, will result in a need for deoxynucleotides. To synthesize deoxynucleotides, there may be an increase in the protein levels of ribonucleotide reductase. Primary human chronic lymphocytic leukemia (CLL) cells were used as a quiescent cell model system. The cells were treated with 4-hydroperoxycyclophosphamide (4-HC) to induce DNA damage. Incubation with 4-HC at 3, 10, and 50 μM for up to 6 hours resulted in dose- and time-dependent increase in unscheduled DNA synthesis (UDS), as measured by [^{3}H]thymidine incorporation. Parallel determinations in these cells demonstrated a median 1.6 fold (range 1.0 – 2.5) increase in the expression level of the M2 subunit of RNR. In contrast, protein levels of M1 subunit of RNR were unaltered. The variability of responses among patients in the M2 subunit levels led us to examine the correlation between the RNR expression and UDS. There was a linear correlation between M2 protein expression and [^{3}H]thymidine incorporation (r = 0.91, p = 0.002) in CLL cells exposed to 10 μM 4-HC for 4 h (n = 1 These results suggest a potential role of RNR in the DNA repair process in CLL cells exposed to alkylating agents. To assess if this response was at transcriptional or translational level, we are assaying the messenger RNA levels of the enzym

#2663 Dicoumarol and allopurinol effects on MMC-induced toxicity in CHO DNA repair deficient cell lines. Pritsos, Karen L., Briggs, Laura A. and Pritsos, Chris A. *Dept of Nutrition and Environmental Sciences and Health Program, Univ. of Nevada, Reno, NV 89557.*

Mitomycin C (MMC) is a clinically important anticancer agent which requires bioreductive activation in order to exert its toxicity. Enzymes involved in MMC activation include DT-diaphorase, xanthine oxidase, xanthine dehydrogenase, cytochrome b5 reductase and NADPH-cytochrome c reductase. MMC's purported antineoplastic mechanism of action involves drug-DNA interactions. A series of DNA repair deficient CHO cells previously developed have varying sensitivities to MMC. The various cell lines are deficient in different types of DNA repair. The mutant CHO cell lines, UV5, UV20 and UV41 are derived from AA8 CHO cells and have the following relative sensitivities to MMC (UV41 > UV20 > UV5 > AA8). We determined the DT-diaphorase, xanthine oxidase and xanthine dehydrogenase activities in these cell lines. We subsequently determined MMC toxicity in these various cell lines in the presence of the DT-diaphorase inhibitor dicoumarol and the xanthine oxidase/dehydrogenase inhibitor allopurinol. Allopurinol did not provide any protection from MMC toxicity. The addition of dicou

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Enhancement of human recombinant tumor necrosis factor (rTNF) effects on a rat glioma model by concomitant injection of murine recombinant interleukin-1 (rIL-1).

Wright, J.L., Merchant, R.E. Medical College of Virginia, Richmond, VA 23298

We administered human rTNF (Cetus) intratumorally either alone or in combination with rIL-1 (DuPont) to investigate any amplifying effects rIL-1 may have on rTNF's anti-tumor activity. Syngeneic RT-2 glioma cells were injected into a parietal lobe Fischer 344 rats. Animals received a single 5 ul stereotaxic injection of $6x10^4$U rTNF plus 2.0 ug IL-1 (T+I), excipient (T+I-E), or $6x10^4$U rTNF alone. After 9 days, models received either T+I, T+I-E, rTNF or no treatment. T+I significantly increased weight loss and temperature increase one day post-injection; weight loss was only significant in T+I models, averaging 12.5% of initial body weight while rTNF and T+I-E recipients did not differ significantly from untreated controls. Both T+I and rTNF injections significantly raised core body temperature; however the T+I effect was higher than rTNF, 1.15°C vs .8°C, respectively. All other models showed either no increase or a decrease of up to 2°C in mean body temperature. Histological studies of the injection site of T+I recipients indicated an increase in hemorrhagic areas and leukocytic infiltration at levels equal to or surpassing those seen in rTNF IC injections and exceeding those seen in untreated or excipient-treated models. These preliminary studies suggest an additive or synergistic role in local and systemic toxicity and anti-glioma effect of rTNF and rIL-1 when administered together.

1748

Interferon-γ (IFN-γ) enhances the cytolytic activity of tumor necrosis factor (TNF) and decreases TNF-inhibitory protein (TIP). Dembinski, W.E., and Ip, M.M. Roswell Park Cancer Institute, 666 Elm St., Buffalo, NY 14263.

Various studies have demonstrated that IFN-γ increases the antitumor activity of TNF. We have observed that IFN-γ increases the cytolytic effect of TNF on human colon adenocarcinoma HT-29 and human bladder transitional-cell papilloma RT4 cells even when IFN-γ was added to the cells after removal of TNF. This suggests that processes other than upregulation of the TNF receptor may account for the synergism of these agents. In an attempt to establish the mechanism of this synergy, we assayed the level of production of TIP by human peripheral blood leukocytes (PBL), as well as by several cell lines. TIP has been isolated by us from HLD cells and PBL. It is a 26-28 kDa protein which decreases the susceptibility of cells to the cytolytic activity of TNF. IFN-γ reduced production of TIP by all cell lines examined, suggesting that IFN-γ may increase the sensitivity of a target cell to the cytolytic effect of TNF by decreasing production of TIP. Supported by CA24538 and Asahi Chem. Co., Ltd.

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Obligatory role of tumor necrosis factor-α in antibody dependent cellular cytotoxicity by interleukin-2 stimulated peripheral blood lymphocytes.

Lapco-Deenadayalan, S., and Grimm, E.A.
University of Texas, M.D.Anderson Cancer Center, Houston, TX-77030.

Tumor Necrosis Factor-α plays a pivotal role in generation of antibody dependent cellular cytotoxicity (ADCC) by PBL. The kinetics of development of maximal ADCC potential by PBL cultured under various conditions (in IL-2 alone, in IL-2 + TNF, and in TNF alone) were studied. The target cells used were human melanoma cell lines, SK-Mel-1 and Mel RP and the anti-tumor monoclonal antibody used was 140.2a, which recognizes GD2 epitopes. While PBL cultured in IL-2 alone mediated maximum ADCC after 24 hours of culture, addition of TNF to activation cultures resulted in enhanced ADCC which persisted for as long as 72 hours. Surprisingly, culture of PBL in TNF alone also enhanced ADCC for up to 48 hours. The stoichiometry of Fc receptor (FcR) expression on effectors did not reveal any correlation to the ADCC exhibited by them. However, preincubation of the effectors with anti-FcR antibodies blocked ADCC. Thus, although FcRs are necessary to mediate ADCC, other factors including TNF and/or IL-2, regulate the degree of ADCC mediated. Neither exogenous TNF nor anti-TNF antibodies added at the time of the cytotoxicity assay had any effect on ADCC. However, addition of anti-TNF antibodies to PBL during activation culture with IL-2, resulted in marked inhibition of the ADCC, demonstrating the crucial role of TNF throughout the activation phase of ADCC effectors.

1750

Enhanced Immunogenicity of a Non-H2 Expressing Murine Tumor That is Producing IL-2.

Frost, P., Hunt, B., and Itaya, T. The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

CBA-SP1 murine adenocarcinoma cells transfected with the IL-2 gene (SP1/IL-2) express 20-25,000 U/ml of IL-2. While the parent SP1 cells are highly tumorigenic (Tum^+), SP1/IL-2 cells fail to grow in syngeneic hosts. SP1/IL-2 cells engender a CTL response that is inhibited by anti-CD8 antibody *in vitro*. All SP1 cells express Class II MHC antigens but unlike SP1/IFN-γ cells, SP1/IL-2 cells do not express Class I MHC antigens. SP1/IL-2 are similar to SP1 cells expressing a viral gene (HA) in that they are IMM^+ but are not able to protect against a challenge with parental SP1 cells. Protection seems to require the expression of Class I MHC antigens. The role of paracrine lymphokine production by tumor cells in tumor rejection will be discussed.

1751

Transcription and secretion of TNF and IL-1 from monocytes activated by liposome-encapsulated Muramyl Tripeptide.

Maeda, N., Knowles, R.D., and Kleinerman, E.S. U.T.M.D. Anderson Cancer Center, Houston, Texas 77030.

Muramyl tripeptide phosphatidylethanolamine (MTP-PE), an analogue of MDP, can be incorporated into liposomes (L-MTP-PE). L-MTP-PE *in vitro* stimulated monocytes to selectively kill tumor cells. Furthermore, the activation of monocyte tumoricidal function was demonstrated following the i.v. infusion of L-MTP-PE in patients. The purpose of this study was to determine the mechanism by which L-MTP-PE activates monocytes. Monocyte tumoricidal function is linked to both IL-1 and TNF. Therefore, normal human monocytes were incubated for with L-MTP-PE, empty liposomes, or medium. The supernatants were removed and assayed for TNF and IL-1. TNF was detected after 4 h incubation with L-MTP-PE, but not empty liposomes or medium. TNF secretion peaked at 8 h and decreased by 72 h. This increased TNF was associated with an 8-fold increase in TNF mRNA. IL-1 secretion was detected after 8 h, peaked by 24 h and decreased by 48 h. These data indicate that L-MTP-PE activates monocyte tumoricidal function through transcriptional enhancement of TNF (possibly IL-1) and subsequent secretion of both IL-1 and TNF.

1752

Inhibitors of poly(ADP-ribose)polymerase modulate the resistance of SKOV3 cells to the cytotoxic and DNA-degradative effects of tumor necrosis factor. Lichtenstein, A., Andrews, J., and Ware, C.F. VA Wadsworth-UCLA Med. Ctr. and Univ. of Cal. at Riverside, Los Angeles and Riverside, CA. 90073.

The mechanism of resistance to the cytotoxic action of tumor necrosis factor (TNF) was investigated in SKOV3 ovarian cancer cells which over-express HER2 oncogenes. The TNF receptor on SKOV3 cells demonstrated normal binding parameters (Kd=0.6nM and 900 sites/cell). Resistance to TNF was not reversed by inhibitors of protein synthesis or the glutathione cycle. Although SKOV3 cells showed no detectable DNA strand breaks during exposure to TNF, activation of the enzyme poly(ADP-ribose)polymerase (PARP) occurred which was significantly greater than that seen in TNF-sensitive L929 targets. Since PARP has been implicated in DNA repair, we tested whether efficient repair in SKOV3 cells participated in resistance to TNF. Aminobenzamide (ABA) and nicotinamide, 2 inhibitors of PARP, sensitized SKOV3 cells to TNF-mediated cytotoxicity in a concentration-dependent fashion concurrent with the induction of DNA strand breakage. In contrast, ABA diminished TNF cytotoxicity of L929 cells. The data suggest proficient DNA repair is one mechanism by which tumor cells can resist TNF-induced cytotoxicity.

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Interactive effects of inhibitors of poly(ADP-ribose) polymerase and DNA-dependent protein kinase on cellular responses to DNA damage

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DNA-dependent protein kinase (DNA-PK) and poly(ADP-ribose) polymerase (PARP) are activated by DNA strand breaks and participate in DNA repair. We investigated the interactive effects of inhibitors of these enzymes [wortmannin (WM), which inhibits DNA-PK, and 8-hydroxy-2-methylquinazolin-4-one (NU1025), a PARP inhibitor] on cell survival and DNA double-strand break (DSB) and single-strand break (SSB) rejoining in Chinese hamster ovary-K1 cells following exposure to ionizing radiation (IR) or temozolomide. WM (20 μM) or NU1025 (300 μM) potentiated the cytotoxicity of IR with dose enhancement factors at 10% survival (DEF_{10}) values of 4.5 ± 0.6 and 1.7 ± 0.2, respectively. When used in combination, a DEF_{10} of 7.8 ± 1.5 was obtained. WM or NU1025 potentiated the cytotoxicity of temozolomide, and an additive effect on the DEF_{10} value was obtained with the combined inhibitors. Using the same inhibitor concentrations, their single and combined effects on DSB and SSB levels following IR were assessed by neutral and alkaline elution. Cells exposed to IR were post-incubated for 30 min to allow repair to occur. WM or NU1025 increased net DSB levels relative to IR alone (DSB levels of 1.29 ± 0.04 and 1.20 ± 0.05, respectively, compared with 1.01 ± 0.03 for IR alone) and the combination had an additive effect. WM had no effect on SSB levels, either alone or in combination with NU1025. SSB levels were increased to 1.27 ± 0.05 with NU1025 compared with IR alone, 1.02 ± 0.04. The dose-dependent effects of the inhibitors on DSB levels showed that they were near maximal by 20 μM WM and 300 μM NU1025. DSB repair kinetics were studied. Both inhibitors increased net DSB levels over a 3 h time period; when they were combined, net DSB levels at 3 h were identical to DSB levels immediately post-IR. The combined use of DNA repair inhibitors may have therapeutic potential.

Introduction

Ionizing radiation (IR) produces a complex variety of lesions in the DNA which give rise to DNA single-strand breaks (SSBs) and double-strand breaks (DSBs), either by chemical decomposition following free radical attack or as a result of the early steps of DNA repair pathways. Two important enzymes, poly(ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK), bind to, and are activated by, these DNA breaks (for reviews, see refs 1–3). Mutant cell lines that are defective in either the catalytic subunit (DNA-PKcs) or one of the DNA binding subunits (e.g. Ku80) of DNA-PK are unable to repair IR-induced DNA DSBs, are defective in V(D)J recombination and are highly radiosensitive (e.g. refs 4,5). The fungal metabolite, wortmannin (WM), inhibits DNA-PK and thereby inhibits DSB repair and potentiates IR-induced cytotoxicity (6–8). Prevention of DSB rejoining by WM has also been demonstrated in cell-free extracts, thus substantiating the direct effect of WM on DSB rejoining (9). Although WM is not a specific inhibitor of DNA-PK, as it also inhibits phosphatidylinositol 3-kinase (PI 3-K) and may potentially inhibit the ataxia telangiectasia gene product (ATM) (both of which share active site homology with DNA-PK) (10,11), its use has identified DNA-PK as a potential target for developing drugs that sensitize cells to IR via inhibition of DNA repair.

Potent PARP inhibitors have already been developed with this aim in mind (12–14), and have been shown to potentiate the cytotoxicity of alkylating agents and IR. For example, Boulton *et al.* (15) demonstrated that the PARP inhibitor 8-hydroxy-2-methylquinazolin-4-one (NU1025) potentiated the cytotoxicity of the monofunctional alkylating agent temozolomide and this correlated with an inhibition of SSB repair.

To date, a large body of evidence has pointed to an involvement of PARP function in the base excision repair pathway, which generates SSBs as repair intermediates. Indeed, the observations of potentiation of the cytotoxicity of DNA damaging agents and inhibition of SSB repair in early studies using inhibitors (e.g. ref. 16) have now been finally confirmed in PARP-deficient cell lines (17). However, the possible function of PARP in DSB repair has been largely neglected, although Benjamin and Gill originally demonstrated in 1980 that PARP was activated by DSBs as well as SSBs (18), and this has more recently been confirmed by Weinfield *et al.* (19) who showed, using highly purified enzymes, that DSBs activated PARP with almost equal efficiency as SSBs but that DNA-PK could only be activated by DSBs. Moreover, two reports have shown that the rejoining of DSBs induced by the electroporation of restriction enzymes into cells was delayed by the classical PARP inhibitor, 3-aminobenzamide (20,21).

The aim of this study was to investigate the single and combined effects of NU1025 and WM on cytotoxicity and DNA damage repair induced by IR and temozolomide in cell culture. The results provide promising prospects for enhancing the efficacy of radiotherapy and temozolomide via the combined inhibition of mechanistically diverse DNA repair enzymes.

Materials and methods

Materials

WM was obtained from Sigma (St Louis, MO). It was dissolved in anhydrous dimethyl sulfoxide (DMSO) at a stock concentration of 10 mM and stored

Abbreviations: DEF_{10}, dose enhancement factor at 10% survival; DMSO, dimethyl sulfoxide; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; IR, ionizing radiation; PARP, poly(ADP-ribose) polymerase; RR, relative retention; SSB, single-strand break; WM, wortmannin.

at −20°C. NU1025 was provided by the Newcastle upon Tyne Anticancer Drug Development Initiative (ADDI) (Newcastle upon Tyne, UK) and its synthesis has been described elsewhere (13). Temozolomide was a gift from Professor M.F.G.Stevens (Cancer Research Laboratories, University of Nottingham, UK). NU1025 and temozolomide stock solutions were prepared in DMSO at 100 mM. Solvent concentrations in cell culture experiments were kept constant and at <1% by appropriate additions of DMSO.

Cell culture

CHO-K1 cells were maintained as monolayers in RPMI 1640 medium (supplemented with 10% fetal calf serum, glutamine and antibiotics). HEPES and sodium bicarbonate were added at final concentrations of 18 and 11 mM, respectively. Clonogenic assays were performed as previously described (6). Briefly, cells as monolayers were preincubated ± WM ± NU1025 for 1 h prior to exposure to IR, then post-incubated for 16 h. Cells were then trypsinized and replated for survivors in the absence of drugs. Similarly, following a 1 h incubation with inhibitor(s), cells were treated with temozolomide for 16 h and then trypsinized and replated as above. The data are averaged from at least three independent experiments ± SE. The dose enhancement factors at 10% survival (DEF_{10}) were calculated from the survival curves by taking the ratio of the dose of IR that reduced survival to 10% divided by the dose of IR that reduced survival to 10% in the presence of inhibitor(s).

DNA strand break assays

The filter elution techniques for assaying DSB and SSB levels have been described in detail (22,23), and the radiolabelling, drug treatment, post-incubation conditions and sample preparation used in these experiments were identical to those described by Boulton *et al.* (6). In all experiments, cells were exposed to either 6 Gy (SSB assay) or 100 Gy (DSB assay). Cell cultures were preincubated ± NU1025 ± WM for 1 h prior to exposure to IR, and the compounds remained in the culture medium during the post-incubation periods. SSB and DSB levels were quantitated as follows. The relative retention (RR) value is the fraction of sample DNA retained on the filter when 50% of the internal standard has eluted. The RR values of DSBs and SSBs in cells treated with inhibitor(s) were expressed relative to the RR values for cells treated with IR alone (Figures 2 and 3) or to the RR value of unirradiated cells (Figure 4). In each case, the RR value of the 'control' cells was normalized to 1.0, and the sample RR values proportionated accordingly. Thus, a DNA strand break level of 1.0 indicates that there is no difference in DNA strand break levels between the designated 'control' cells and sample cells treated with inhibitor(s). Data points represent the mean of at least four independently dosed samples from two or more separate experiments ± SE.

Results

Radiosensitization and chemopotentiation by WM and NU1025

The effects of WM and NU1025 on IR-induced cytotoxicity were investigated. WM (20 μM) or NU1025 (300 μM) alone, neither of which caused loss of clonogenic survival (either *per se* or in combination), potentiated the cytotoxicity of IR (Figure 1). When used in combination, at least additive effects on cytotoxicity were observed. The DEF_{10} values for a range of inhibitor concentrations and combinations are summarized in Table I. Note the very large DEF_{10} value (7.8 ± 1.5) obtained for the combination of WM (20 μM) + NU1025 (300 μM). Similar experiments were performed using temozolomide as the cytotoxic agent, and the results are summarized in Table II. Again, both WM and NU1025 potentiated the cytotoxicity of temozolomide and the combination of inhibitors produced approximately additive effects on the DEF_{10} values.

DNA strand break levels

SSB and DSB levels were assessed in inhibitor-treated cells 30 min post-IR. By this time we have previously established that the majority of DNA strand break rejoining has occurred (6). The results are presented as a histogram in Figure 2. WM (20 μM) and NU1025 (300 μM) increased relative DSB levels from 1.01 ± 0.03 for IR alone to 1.29 ± 0.04 and 1.20 ± 0.05, respectively (Figure 2A). When the inhibitors were combined, relative DSB levels increased to 1.61 ± 0.03. In marked contrast, when SSB levels were assessed, WM alone had no effect on SSB levels (0.98 ± 0.04 compared with 1.02 ± 0.04 for IR alone) (Figure 2B). NU1025 increased SSB levels to 1.27 ± 0.05 and this value was not changed significantly by co-incubation with WM.

Although WM potentiated the cytotoxicity of temozolomide, it was not possible to detect DSBs by neutral elution, even at concentrations of temozolomide as high as 1 mM. We have previously established (15) that NU1025 increases temozolomide-induced SSB levels and therefore no further investigations of the inhibitors on temozolomide-induced DNA strand break production were undertaken here.

A further study comprised a comparison of the dose-dependent effects of the inhibitors on DSB levels 30 min post-IR. The results are shown in Figure 3. Both WM and NU1025 increased DSB levels in a dose-dependent manner (Figure 3A

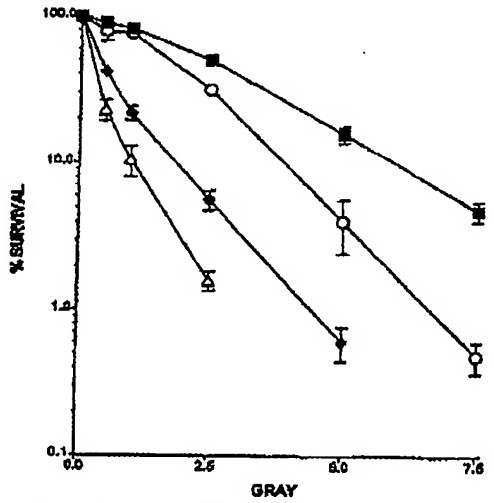

Fig. 1. Effects of increasing doses of IR on clonogenic survival in the presence or absence of WM and NU1025. ■, IR alone; ◆, IR+WM (20 μM); ○, IR + NU1025 (300 μM); △, IR + WM (20 μM) + NU1025 (300 μM).

Table I. Comparison of the DEF_{10} values derived from IR survival curves using a range of inhibitor concentrations and combinations

WM (μM)	NU1025		
	0 μM	100 μM	300 μM
0	1.0 ± 0.1	1.1 ± 0.1	1.7 ± 0.2
5	2.3 ± 0.3	3.1 ± 0.3	Not done
20	4.5 ± 0.6	5.9 ± 0.7	7.8 ± 1.5

Table II. Comparison of the DEF_{10} values derived from temozolomide survival curves using a range of inhibitor concentrations and combinations

WM (μM)	NU1025	
	0 μM	100 μM
0	1.0 ± 0.3	2.6 ± 0.6
20	2.6 ± 0.2	4.3 ± 0.7

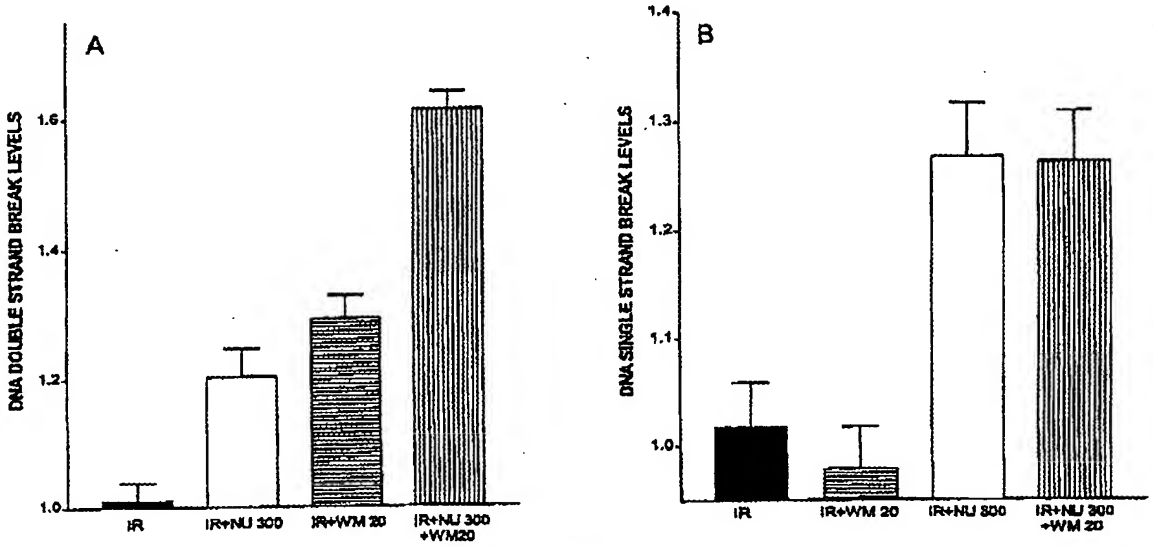

Fig. 2. Effects of WM and/or NU1025 on DSB and SSB levels. Cells were exposed to IR (100 Gy for DSB, and 6 Gy for SSB assays), in the presence or absence of WM (20 μM) and NU1025 (300 μM). Cells were post-incubated for 30 min before harvesting for elution assays. (A) DSB assay; (B) SSB assay.

and B, respectively), but 300 μM NU1025 was required to achieve an increase comparable to 20 μM WM.

Kinetics of DSB repair

The effects of WM and NU1025 on the kinetics of DSB repair following exposure to IR were compared over a 3 h time period and the results are shown in Figure 4. In the absence of the inhibitors, DSBs were rejoined rapidly with the majority rejoined by 60 min. Although a small amount of DSB rejoining initially occurred during the first 30 min in the presence of WM (50 μM), DSB levels subsequently increased up to 180 min post-irradiation (1.53 ± 0.04 for IR + WM compared with 1.04 ± 0.01 for IR alone) (Figure 4). The production of these additional DSBs was not attributable to a direct effect of WM on the integrity of DNA, as we have previously shown that prolonged incubation with WM alone did not cause DSB formation (6). A possible explanation for the formation of additional DSBs observed, in particular since supralethal doses of IR (100 Gy) have to be used to detect DSBs by neutral elution, is the early onset of DNA fragmentation associated with apoptosis. Finally, the inhibitors were combined, in this case with the WM added prior to exposure to IR, and NU1025 added immediately afterwards to preclude possible interactive effects of the drugs on DSB production during IR exposure. (We have found it necessary to add WM prior to exposure to IR to obtain optimum inhibition of DSB repair.) In this case, approximately additive effects on DSB levels were seen throughout the 3 h time period (Figure 4) such that by 180 min the net level of DSBs was about the same as immediately post-IR, compared with the almost complete rejoining observed in the absence of inhibitors.

Discussion

As mentioned in the Introduction, molecular evidence indicates that PARP interacts with DSBs as well as SSBs. PARP has two zinc fingers, both of which are required for SSB binding, but the first alone suffices to bind PARP to a DSB, which also acts as a more potent activator of PARP than a SSB (24). Chung *et al.* (20) showed that 3-aminobenzamide increased chromosomal aberrations and retarded repair of DNA damage resulting from the electroporation of restriction enzymes into cells. Bryant and Johnston (21) also demonstrated an effect of PARP inhibitors on the repair of restriction enzyme-induced DSBs. Numerous publications have shown that PARP is involved in sister chromatid exchanges and gene amplification. These observations have led to the proposal that PARP may function to prevent spurious recombination events at DSBs in the DNA (2).

The data presented here clearly demonstrate that inhibition of PARP, as well as DNA-PK, retards DSB rejoining. We have considered the possibility that the effect of PARP inhibition in increasing DSB levels could be an artefact of the neutral elution assay allowing the detection of a low level of SSBs, since they are the predominant lesions produced in irradiated DNA. If this were the case, the effect of NU1025, by increasing net SSB levels, would be to apparently increase DSB levels. However, it has been clearly demonstrated that excess SSBs do not interfere with the DSB assay used here (25).

The additional DSBs, as defined by the neutral elution technique, obtained in the presence of NU1025 may arise because of a retardation of DSB rejoining. Alternatively, they may arise from a subset of IR-induced lesions being converted to DSBs. For example, proximal SSBs on complementary strands could be stabilized and repaired by a two-step SSB repair process when PARP is functioning; when PARP is inhibited, these could convert to DSBs. This is a distinct possibility since IR produces localized clusters of multiple damages, which in addition to producing DSBs by direct chemical reaction, will have the potential to convert to DSBs during attempts at repair (26,27).

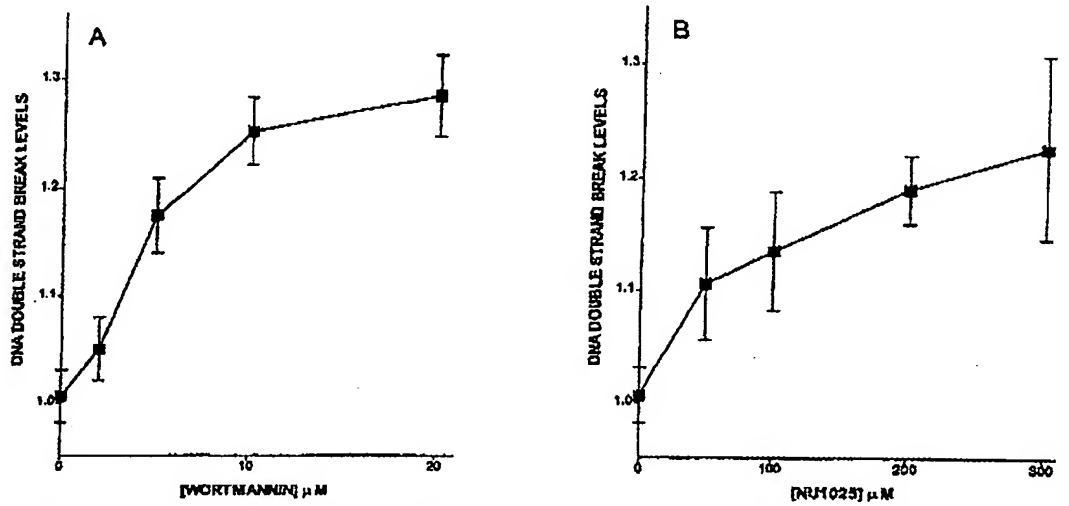

Fig. 3. Dose-dependent effects of inhibitors on DSB levels. Cells were exposed to 100 Gy IR, and post-incubated for 30 min to allow repair to occur before harvesting cells for neutral elution. (A) Dose-dependent effects of WM; (B) dose-dependent effects of NU1025.

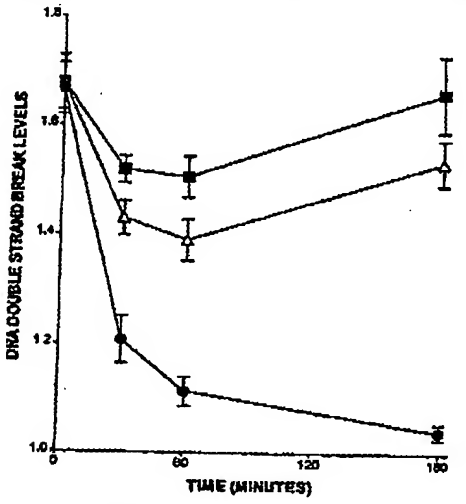

Fig. 4. Kinetics of DSB repair. Cells ± inhibitors were pre-incubated with WM for 1 h, exposed to 100 Gy IR, NU1025 added, and post-incubated for increasing lengths of time before harvesting for neutral elution. ●, IR alone; △, IR + WM 150 μM; ■, IR +WM (50 μM) + NU1025 (300 μM).

WM is known to inhibit PI 3-K as well as DNA-PK, and may also inhibit other members of this family of kinases, including the ataxia telangiectasia gene product, ATM, or ATR (for ATM and Rad3-related) (11,28). Mutant ATM cell lines, or ATR cell lines over-expressing kinase-inactive ATR protein, demonstrate similar hypersensitivity to IR as DNA-PK defective cell lines (29,30). Therefore, although WM clearly inhibits DNA-PK and inhibits DNA-PK mediated DSB repair (7–9), the ability of WM to potentiate IR-induced cytotoxicity could also be mediated, at least in part, via an inhibition of ATM or ATR. The development of specific assays for these enzymes will be required to address these issues. However, we have demonstrated (unpublished data) that potentiation of IR-induced cell killing by WM is largely abolished in the *xrs-6* cell line, which is mutated in the Ku80 subunit of DNA-PK (31), supporting the contention that the effects of WM on the cytotoxic response are caused by DNA-PK inhibition.

Although WM potentiated the cytotoxicity of temozolomide, no DSBs were detectable by neutral elution. However, this chemopotentiation is still consistent with an inhibition of DNA-PK, since known DNA-PK defective mutant cell lines have been demonstrated to be hypersensitive to other monofunctional alkylating agents (32). It is probable that inhibition of the repair of very low levels of DSBs, below the relatively insensitive detection limits of the neutral elution assay, would suffice to enhance temozolomide cytotoxicity. As with IR, a useful additive effect on the DEP_{10} value was obtained when the inhibitors were combined. It should be stressed that although we have shown that NU1025 modulates DSB repair, it also retards SSB repair in IR- and temozolomide-treated cells, and hence it is not possible to ascribe its potentiating effects on cytotoxicity to a single repair pathway.

These data point to cooperation between PARP and DNA-PK at direct DSBs or DSBs that are formed as repair intermediates. As well as regulating DSB and SSB repair in a very similar manner (compare the effects of NU1025 on the kinetics of DSB rejoining presented here with its effects on the kinetics of SSB repair [15]), PARP may also function to promote DNA-PK-mediated non-homologous end-joining by preventing DSB repair by an alternative pathway involving homologous recombination. A role for PARP as an anti-recombinogenic factor has been proposed (2), and this hypothesis is consistent with recent evidence that an additional loss of PARP function in DNA-PK deficient mice can rescue the block in V(D)J recombination that typifies the *SCID* phenotype (33). An alternative hypothesis is that PARP may function directly to

activate DNA-PK, and good evidence for this has recently been published. Ruscetti *et al.* (34) have shown, using purified enzymes, that the kinase activity of DNA-PK is stimulated by poly(ADP-ribosylation) of its catalytic subunit.

Mice lacking PARP and/or DNA-PK are viable (33,35,36), which is an important consideration in radio- and chemotherapy, as specific inhibitors of these enzymes should therefore exhibit no systemic toxicity. The most recently developed PARP inhibitors include 2-(4-methoxyphenyl)benzimidazole-4-carboxamide, synthesized as part of the programme of the Newcastle upon Tyne ADDI group. This compound has an IC_{50} value for inhibition of PARP of 0.06 μM, compared with 0.4 μM for NU1025, and is probably of sufficient potency to be active at physiologically achievable concentrations (14). The evident effectiveness and potency of WM acting as a DNA-PK inhibitor, either alone or in conjunction with NU1025, to potentiate IR- and temozolomide-induced cytotoxicity, indicates that DNA-PK represents another valid repair enzyme target for drug development. The additive effects of the two repair inhibitors on the cytotoxicity of IR and temozolomide may prove powerful tools to enhance their efficacy in cancer therapy.

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References

1. de Murcia,G. and Ménissier de Murcia,J. (1994) Poly(ADP-ribose) polymerase: a molecular nick-sensor. *Trends Biochem. Sci.*, 19, 172–176.
2. Lindahl,T., Satoh,M.S., Poirier,G.G. and Klungland,A. (1995) Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. *Trends Biochem. Sci.*, 20, 405–411.
3. Jackson,S.P. (1996) DNA damage detection by DNA dependent protein kinase and related enzymes. *Cancer Surv.*, 28, 261–279.
4. Jeggo,P.A., Taccioli,G.E. and Jackson,S.P. (1995) Ménage a trois: double strand break repair, V(D)J recombination and DNA-PK. *Bioessays*, 17, 949–957.
5. Roth,D.B., Lindahl,T. and Gellert,M. (1995) How to make ends meet. *Current Biol.*, 5, 496–499.
6. Boulton,S., Kyle,S., Yalgintepe,L. and Durkacz,B.W. (1996) Wortmannin is a potent inhibitor of DNA double strand break repair but not single strand break repair in Chinese hamster ovary cells. *Carcinogenesis*, 17, 2285–2290.
7. Price,B.D. and Youmell,M. (1996) The PI 3-kinase inhibitor wortmannin sensitizes murine fibroblasts and human tumour cells to radiation and blocks induction of p53 following DNA damage. *Cancer Res.*, 56, 246–250.
8. Rosenzweig,K.E., Youmell,M.B., Palayoor,S.T. and Price,B.R. (1997) Radiosensitization of human tumour cells by the phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 correlates with inhibition of DNA-dependent protein kinase and prolonged G2-M delay. *Clin. Cancer Res.*, 3, 1149–1156.
9. Gu,X.-Y., Bennet,R.A.O. and Porvik,L.F. (1996) End-joining of free radical-mediated DNA double-strand breaks *in vitro* is blocked by the kinase inhibitor wortmannin at a step preceding removal of damaged 3′ termini. *J. Biol. Chem.*, 271, 19660–19663.
10. Powis,G., Bonjouklian,R., Berggren,M.M. *et al.* (1994) Wortmannin, a potent and selective inhibitor of phosphatidylinositol 3-kinase. *Cancer Res.*, 54, 2419–2423.
11. Hartley,K.O., Gell,D., Smith,G.C.M. *et al.* (1995) DNA-dependent protein kinase catalytic subunit: a relative of phosphatidylinositol 3-kinase and the ataxia telangiectasia gene product. *Cell*, 82, 849–856.
12. Suto,M.J., Turner,W.R., Arundel-Suto,C.M., Werbel,L.M. and Sebolt-Leopold,J.S. (1991) Dihydroisoquinolinones: the design and synthesis of a new series of potent inhibitors of poly(ADP-ribose) polymerase. *Anticancer Drug Des.*, 7, 101–107.
13. Griffin,R.J., Pemberton,L.C., Rhodes,D. *et al.* (1995) Novel potent inhibitors of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP). *Anticancer Drug Des.*, 10, 507–514.
14. Griffin,R.J., Srinivasan,S., White,A.W., Bowman,K., Calvert,A.H., Curtin,N.J., Newell,D.R. and Golding,B.T. (1996) Novel benzimidazole and quinazolinone inhibitors of the DNA repair enzyme, poly(ADP-ribose) polymerase. *Pharmaceut. Sci.*, 2, 43–47.
15. Boulton,S., Pemberton,L.C., Porteous,J.K., Curtin,N.J., Griffin,R.J., Golding,B.T. and Durkacz,B.W. (1995) Potentiation of temozolomide-induced cytotoxicity: a comparative study of the effects of poly(ADP-ribose) polymerase inhibitors. *Br. J. Cancer*, 72, 849–856.
16. Durkacz,B.W., Omidiji,O., Gray,D.A. and Shall,S. (1980) (ADP-ribose)$_n$ synthesis participates in DNA repair. *Nature*, 283, 593–596.
17. Trucco,C., Oliver,F.J., de Murcia,G. and Menissier-de Murcia,J. (1998) DNA repair defect in poly(ADP-ribose) polymerase-deficient cell lines. *Nucleic Acids Res.*, 26, 2644–2649.
18. Benjamin,R.C. and Gill,D.M. (1980) Poly(ADP-ribose) synthesis *in vitro* programmed by damaged DNA. A comparison of DNA molecules containing different types of strand breaks. *J. Biol. Chem.*, 255, 10502–10508.
19. Weinfeld,M., Chaudhry,M.A., D'Amours,D., Pelletier,J.D., Poirier,G.G., Povirk,L.F. and Lees-Miller,S.P. (1997) Interaction of DNA-dependent protein kinase and poly(ADP-ribose) polymerase with radiation-induced DNA strand breaks. *Radiat. Res.*, 148, 22–28.
20. Chung,H.W., Philips,J.W., Winegar,R.A., Preston,J. and Morgan,W.F. (1991) Modulation of restriction enzyme-induced damage by chemicals that interfere with cellular responses to DNA damage: a cytogenic and pulsed-field gel analysis. *Radiat. Res.*, 125, 107–113.
21. Bryant,P.E. and Johnston,P.J. (1993) Restriction-endonuclease-induced DNA double-strand breaks and chromosomal aberrations in mammalian cells. *Mutat. Res.*, 299, 289–296.
22. Kohn,K.W., Ewig,R.A.G. and Zwelling,L.A. (1981) Measurement of strand breaks and crosslinks by alkaline elution. In Friedberg,E.C. and Hanawalt,P.C. (eds) *DNA Repair: A Laboratory Manual of Research Procedures*, Marcel Dekker, New York and Basel, vol. 2, part B, pp. 379–401.
23. Bradley,M.O. and Kohn,K.W. (1979) X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. *Nucleic Acids Res.*, 7, 793–804.
24. Ikejima,M., Noguchi,S., Yamashita,R., Ogura,T., Sugimura,T., Gill,D.M. and Miwa,M. (1990) The zinc fingers of human poly(ADP-ribose) polymerase are differentially required for the recognition of DNA strand breaks and nicks and consequent enzyme activation. *J. Biol. Chem.*, 255, 21907–21913.
25. Johnston,P.J. and Bryant,P.E. (1991) Lack of interference of DNA single-strand breaks with the measurement of double-strand breaks in mammalian cells using the neutral filter elution assay. *Nucleic Acids Res.*, 19, 2735–2738.
26. Ward,J.F. (1994) The complexity of DNA damage: relevance to biological consequences. *Int. J. Radiat.*, 66, 427–432.
27. Holley,W.R. and Chatterjee,A. (1996) Clusters of DNA damage induced by ionising radiation: formation of short DNA fragments. I. Theoretical modelling. *Radiat. Res.*, 145, 188–199.
28. Cimprich,K.A., Shin,T.B., Keith,C.T. and Schreiber,S.L. (1996) cDNA cloning and gene mapping of a candidate human cell cycle checkpoint protein. *Proc. Natl Acad. Sci. USA*, 93, 2850–2855.
29. Taylor,A.M.R., Harnden,D.G., Arlett,C.F., Harcourt,A.R., Lehmann,A.R., Stevens,S. and Bridges,B.A. (1975) Ataxia telangiectasia, a human mutation with abnormal radiation sensitivity. *Nature*, 258, 427–429.
30. Cliby,W.A., Roberts,C.J., Cimprich,K.A., Stringer,C.M., Lamb,J.R., Schreiber,S.L. and Friend,S.H. (1998) Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. *EMBO J.*, 17, 159–169.
31. Singleton,B.K., Priestly,A., Steingrimsdottir,H., Gell,D., Blunt,T., Jackson,S.P., Lehmann,A.R. and Jeggo,P.A. (1997) Molecular and biochemical characterisation of *xrs* mutants defective in Ku80. *Mol. Cell. Biol.*, 17, 1264–1273.
32. Jeggo,P.A. and Kemp,L.M. (1983) X-ray sensitive mutants of Chinese hamster ovary cell line. Isolation and cross sensitivity to other DNA-damaging agents. *Mutat. Res.*, 112, 313–327.
33. Morrison,C., Smith,G.C.M., Stingl,L., Jackson,S.P., Wagner,E.F. and Wang,Z.-Q. (1997) Genetic interaction between PARP and DNA-PK in V(D)J recombination and tumorigenesis. *Nature Genet.*, 17, 479–482.
34. Ruscetti,T., Lehnert,B.E., Halbrook,J., Trong,H.L., Hockstras,M.P., Chen,D.J. and Peterson,S.R. (1998) Stimulation of DNA-dependent protein kinase by poly(ADP-ribose) polymerase. *J. Biol. Chem.*, 273, 14461–14467.
35. Wang,Z.-Q., Auer,B., Stingl,L., Berghammer,H., Haidacher,D., Schweiger,M. and Wagner,E.F. (1995) Mice lacking ADPRT and poly(ADP-ribosyl)ation develop normally but are susceptible to skin disease. *Genes Dev.*, 9, 509–520.
36. Ménissier de Murcia,J., Niedergang,C., Trucco,C. *et al.* (1997) Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. *Proc. Natl Acad. Sci. USA*, 94, 7303–7307.

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Pharmacologic Disruption of Base Excision Repair Sensitizes Mismatch Repair-deficient and -proficient Colon Cancer Cells to Methylating Agents[1]

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ABSTRACT

Previously we showed that a mismatch repair (MMR)-deficient cell line, HCT116 (hMLH1 mut), unlike a MMR wild-type cell line, SW480, was more resistant to the therapeutic methylating agent, temozolomide (TMZ), because the MMR complex fails to recognize TMZ-induced O^6-methylguanine DNA adduct mispairings with thymine that arise after replication. TMZ also produces N^7-methylguanine and N^3-methyladenine adducts that are processed efficiently by the base excision repair (BER) system. After removal of the methylated base by methylpurine glycosylase, which creates the abasic or apurinic-apyrimidinic (AP) site, the phosphodiester bond is hydrolyzed immediately by AP endonuclease, initiating the repair of the AP site. Methoxyamine (MX) reacts with the abasic site and prevents AP endonuclease cleavage, disrupting DNA repair. MX potentiated the cytotoxic effect of TMZ with a dose modification factor (DMF) of 2.3 ± 0.12 in SW480 and 3.1 ± 0.16 in HCT116. When combined with O^6-benzylguanine (BG),

MX and TMZ dramatically increased TMZ cytotoxicity (65.8-fold) in SW480, whereas no additive effect was seen in HCT116. This suggests that N^7-methylguanine and N^3-methyladenine adducts are cytotoxic lesions in MMR-deficient and wild-type cells when BER is interrupted.

Because poly(ADP-ribose) polymerase (PARP) aids in processing of DNA strand breaks induced during MMR and BER, we asked whether PARP inhibitors would also affect BER-mediated cell killing. We found that PARP inhibitors PD128763, 3-aminobenzimide, and 6-aminonicotinamide increased the sensitivity to TMZ in both HCT116 MMR-deficient cells and SW480 MMR wild-type cells. In HCT116 cells, PD128763 remarkably decreased resistance to TMZ, with a DMF of 4.7 ± 0.2. However, the combination of PD128763, BG, and TMZ had no greater effect, indicating that persistent O^6-methylguanine had no effect on cytotoxicity. In SW480, the DMF for TMZ cytotoxicity was 3.1 ± 0.12 with addition of PD128763 and 36 with addition of PD128763 and BG. Synergy analysis by median effect plots indicated a high degree of synergy between TMZ and MX or PD128763. In contrast, 1,3-bis(2-chloroethyl)-1-nitrosourea combined with either MX or PD128763 showed little if any potentiation observed in the absence of BG in either cell line, suggesting that BER pathway has little impact on cytotoxic processing of 1,3-bis(2-chloroethyl)-1-nitrosourea-induced adducts. These studies indicate that targeting BER with MX or PARP inhibitors enhances the cytotoxicity of methylating agents, even in MMR-deficient cells.

INTRODUCTION

In DNA repair-competent cells, DNA adducts formed by methylating agents may be repaired efficiently or be sites of both mutagenic and cytotoxic damage. In this process, the cellular response is specific for each of the DNA adducts formed. Perhaps the best studied is the response to O^6mG.[3] This adduct may be repaired in a single step reaction by O^6-alkylguanine-DNA (AGT); however, saturation of this protein by an excess of adducts or inhibition by BG results in residual adducts that are both cytotoxic and mutagenic (1). Cytotoxicity results from recognition of this adduct by components of the MMR system, a five- or six-protein complex that recognizes O^6mG:thymine base pairs formed by DNA replication past O^6mG, and excises thymine and surrounding bases, resulting in DNA strand breaks. However, a thymine is preferentially reincorporated opposite the persisting O^6mG, triggering MMR function again. It has been hypothesized that this repetitive aberrant repair process increases DNA double-strand breaks and acts as a trigger of apoptosis (2).

MMR deficiency results in inability to process the O^6mG:T mispair; consequently cells replicate DNA past O^6mG lesions without cell cycle arrest, chromosomal aberrations, or apoptosis and survive in the face of persistent DNA damage (3, 4, 5, 6, 7). The presence of

MMR deficiency in a number of colon cancer cell lines allowed us the opportunity to evaluate the relative contribution of this DNA repair defect in resistance to the methylating chemotherapeutic agent, TMZ. We found that MMR deficiency resulted in 35–60-fold resistance to TMZ in cells defective in either MLH1 or MLH6 even after inhibition of AGT by BG (7).

Although O^6mG is the best studied cytotoxic DNA adduct, it is not the most abundant. TMZ, like other methylating agents, also forms N^7mG and N^3mA DNA adducts at frequencies 11 and 1.5 times that of O^6mG.[4] These DNA adducts are efficiently removed by BER and appear to contribute little to cytotoxicity. In the first step of BER, a series of glycosylases recognize abnormal bases such as N^3mA and N^7mG (8, 9), the T:G mismatch (10), and deaminated bases such as hypoxanthine/oxidized 8-oxo-7,8-dihydroguanine or uracil:A (11, 12, 13, 14). After enzymatic or spontaneous hydrolysis of the N-glycosidic bond and release of the abnormal base, AP endonuclease hydrolyzes the phosphodiester backbone 5' to the lesion and dRpase (a DNA deoxyribophosphodiesterase with activity associated with polymerase [x] {beta}) excises the residual 2-deoxyribose-5-phosphate, generating a gap of one nucleotide. DNA polymerase [x] {beta} fills the gap, and DNA ligase seals the nick. This pathway is called short-patch BER. An alternative pathway for BER involves DNA synthesis to fill a gap of 2 to [x] <= 13 nucleotides. This long-patch repair requires proliferating cell nuclear antigen and proliferating cell nuclear antigen-dependent DNA polymerase (15).

PARP acts as a nick sensor of DNA strand breaks by itself or interaction with XRCC1 and involves in BER. PARP binds damaged DNA, resulting in autoribosylation. The modified protein then releases and allows other proteins to access and repair DNA strand breaks (15, 16, 17). Therefore, PARP participates in BER after nick formation in both short- and long-patch repair. It appears most active in the alternative pathway for BER.

BER as a therapeutic target to increase the cytotoxicity of methylating agents has been documented. Cells deficient in DNA polymerase [x] {beta} or blocked in expression of AP endonuclease by antisense oligonucleotides are sensitized to methylating agents (18, 19). In addition, mice deficient in N^3mA DNA glycosylase exhibited increased sensitivity to alkylating drugs such as BCNU and mitomycin C (20). On the other hand, overexpression of the N^3mA DNA glycosylase, which increases the number of AP sites formed, also increases the cytotoxicity of methylating agents (21). Finally, cells lacking PARP activity are more sensitive to alkylating agents, with increased apoptosis and chromosomal instability (22, 23). These data suggest that balanced expression of proteins in the BER complex is important to the efficient processing of lesions. BER is an important mechanism of resistance to therapeutic methylating agents.

We examined two classes of agents that could inhibit the BER pathway to determine

whether they would increase the cytotoxicity of methylating agents in colon cancer cells, particularly in cells deficient in MMR. Because MMR-deficient cells are tolerant to O^6mG formed by TMZ, any change in cytotoxicity observed after use of a BER inhibitor would be due to interruption in repair of N^7mG and N^3mA DNA adducts. Our first strategy was to combine MX with TMZ. MX has been shown to react with the free aldehyde formed at the abasic site exposed by glycosylases and to reduce cleavage at AP sites in mammalian cells, suggesting that the MX-bound abasic site is not a substrate for AP endonuclease (24) . In the regard that AP sites modified by MX are relatively stable and must be converted to cytotoxic lesions, we hypothesized that MX would interrupt BER in cells and potentiate the cytotoxic effects of TMZ, even in MMR-defective cells. The second strategy we used was to inhibit PARP with PD128763, 3-AB, or 6-AN and to subsequently treat cells with TMZ. We hypothesize that inactivated PARP would affect short- and long-patch BER, destabilize strand breaks, reduce interaction with other proteins during repair of methylated DNA adducts, and lead to cell death, again in both MMR-proficient and -deficient cells.

MATERIALS AND METHODS

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Chemicals and Reagents.
BG was generously provided by Dr. Robert Moschel (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD). Stock solution was made in DMSO. TMZ and BCNU were obtained from the Drug Synthesis and Chemistry Branch, Drug Therapeutic Program, National Cancer Center Institute (Rockville, MD). PD128763 was a gift from Park-Davis Pharmaceutical Division (Ann Arbor, MI). 6-AN, 3-AB, MX, and MMS were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of PD128763, 3-AB, and 6-AN were prepared by dissolving in DMSO and added to cell culture at a final concentration of < 1% DMSO when cells were treated with these compounds. MX was dissolved in sterilized water (pH 7.0). All stock solutions were kept at -20°C. BCNU was prepared fresh in 0.5 ml of 100% ethanol, diluted in PBS, and used within 10 min.

Colony Survival Assay.
SW480 cells were obtained from the American Type Culture Collection, Rockville, MD. HCT116 was obtained from R. Boland, University of Michigan Medical Center (Ann Arbor, MI). All cell lines were cultured in appropriate growth media.

Cells (2000/dish) were plated, adhered for 18 h, and treated with TMZ or MMS plus or minus variable modifiers such as BG, MX, 6-AN, 3-AB, or PD128763, according to experimental protocol. After treatment, cells were washed and fresh medium was added. The cells were grown for a further 7 days prior to staining with methylene blue for determination of colonies containing more than 50 cells. Comparisons of drug-induced

cytotoxicity consisted of a calculation of the DMF, defined as the ratio of the IC_{50} of either TMZ or MMS in the absence of indicated modifier(s) to that in the presence of indicated modifier(s), *i.e.*, DMF = IC_{50} for TMZ alone/IC_{50} for TMZ plus modifier(s). The DMF indicates the degree of potentiation of cytotoxic agents by modulator.

Median Effect Analysis.
Median effect analysis was used to determine the dose-response interactions between TMZ and either MX or PD128763. Drugs were combined at the ratio of the IC_{50} values for either TMZ and MX or TMZ and PD128763 as determined by survival/concentration curves. The combination was compared with the cytotoxicity of each drug alone in every experiment. The combination index was determined from colony-forming assays at increasing levels of cell killing, using an analysis of multiple drug interaction program (Biosoft, Cambridge, United Kingdom) developed based on the method of Chou and Talalay (25) . Combination index values of less than or greater than 1 indicate synergy and antagonism, respectively, whereas a combination index value of 1 indicates additivity of the drugs.

Flow Cytometry for Cell Cycle Distribution Analysis.
For cell cycle analysis, 10^6 cells were plated in 100-mm tissue culture dishes and exposed to MX (6 mM)/PD128763 (100 μM) or MX (6 mM)/PD128763 (100 μM) plus TMZ (300 μM) at 37°C. After 24–72 h of culture, cells were fixed in 80% ethanol and DNA was stained with 20 μg/ml propidium iodide. The DNA fluorescence of propidium iodide-stained cells was measured with an Elite ESP flow cytometer/cell sorter (Coulter, Miami, FL). Cell cycle distribution was analyzed with the Modfit 5.2 program (Verity Software, Topsham, MA) with at least 10,000 cells per data point.

Western Blotting for PARP Cleavage Detection.
Cell extracts were resolved by SDS-PAGE (8% polyacrylamide) in a Bio-Rad minigel apparatus at 150 V for 1 h. Proteins were transferred onto PVDF membranes, using a Bio-Rad mini *Trans*-Blot cell for 1 h at 100 V. The blotted membranes were blocked with 5% dry milk in Tris-buffered saline and then probed for 2 h with anti-PARP antibody C2-10 (Trevigen, Gaithersburg, MD). After three 5-min washes with Tris-buffered saline-Tween 20 (0.05%), the blots were incubated with secondary antibody, antimouse horseradish peroxidase-anti-IgG for 1 h (Amersham Life Science, Arlington Heights, IL). Antibody binding was visualized by the ECL method, according to manufacturer's instructions (Amersham).

RESULTS

MX Potentiates Cytotoxicity of TMZ.
We previously reported the comparative cytotoxicity of TMZ and BG in the SW480 and HCT116 cell lines (7) . To test whether MX would alter TMZ cytotoxicity, we

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treated SW480 and HCT116 with 6 mM MX (IC_{50} for MX alone was 50 μM in SW480 and 28 μM in HCT116 cells) plus TMZ (0–1500 μM) for 2 h, with or without BG to abolish AGT-mediated removal of O^6mG DNA adducts. SW480 cells were moderately resistant to TMZ, with an IC_{50} of 395 μM, which was reduced 14-fold to 28 μM by BG pretreatment. Greater resistance to TMZ was observed in MLH1-defective HCT116 cells, even after inhibition of AGT by BG (TMZ IC_{50}, 950 μM). In both cell lines, MX potentiated the cytotoxic effect of TMZ (Fig. 1)☐ with a DMF of 2.3 ± 0.12 ($P = 0.0002$) in SW480 and 3.1 ± 0.16 ($P < 0.0001$) in HCT116. In SW480 cells, additive effects of MX and BG were noted, (IC_{50} was reduced from 395 μM to 6 μM, and the DMF was 65.8), whereas with HCT116 cells, no effect of BG was seen in the presence or absence of MX.

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Fig. 1. MX increases the sensitivity of colon cancer cells to TMZ. Cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 6 mM MX and/or 10 μM BG for 2 h prior to 2-h exposure to TMZ. *A*, HCT116 cells; *B*, SW480 cells. ({blacksquare}), TMZ alone; ({diamondsuit}), MX plus TMZ; (•), BG plus TMZ; ({blacktriangleup}), BG and MX plus TMZ. Results are the means of at least three separate experiments performed in duplicate. *Bars*, SD.

To further decipher the role of N^3mA and N^7mG DNA adducts in the relative absence of O^6mG, we evaluated the effect of MX on MMS-mediated cytotoxicity. MMS is a methylating agent that produces far fewer O^6mG adducts (0.3%) and a greater proportion of N^3mA (10%) and N^7mG adducts (87%) than TMZ (25) . The IC_{50} of MMS was 0.82 mM in SW480 and 1.4 mM in HCT116 cells. This difference is smaller than the difference in the TMZ IC_{50} (395 *versus* 950 μM) between these cell lines, probably because the low concentration of O^6mG adducts formed by MMS increases the impact of other DNA adducts. After cells were treated with MMS (0–3 mM) plus 6 mM MX for 1 h, the IC_{50} DMFs, compared with MMS alone, were 2.0 ± 0.14 ($P < 0.002$) in SW480 and 2.3 ± 0.17 ($P = 0.002$) in HCT116 (Fig. 2)☐ . These DMFs were similar to that observed with TMZ. Compared with treatment of SW480 with BG plus TMZ (DMF of 14), BG plus MMS induced less enhancement of cytotoxicity (DMF of 6). This is perhaps due to fewer O^6mG adducts formed by MMS; however, even a small number of O^6mG adducts contribute to cytotoxicity in MMR-proficient cells. When MMS was combined with BG and MX, greater than 10-fold potentiation of cytotoxicity at the IC_{50} for TMZ alone was observed in SW480, whereas no increased toxicity over that of the combination of BG, MX, and MMS was seen in HCT116 cells. From these data, we infer that MX had equal ability to interrupt

BER in these two cell lines.

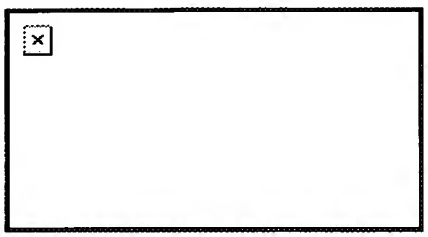

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Fig. 2. Cytotoxicity of MMS enhanced by MX in colon cancer cell lines. Cells were treated with 0–3 mM MMS for 1 h or MMS plus 6 mM MX and/or 10 μM BG for 2 h prior to 2-h exposure to MMS. *A*, HCT10016 cells; *B*, SW480 cells. ({blacksquare}), MMS alone; ({diamondsuit}), MX plus MMS; (•), BG plus MMS; ({blacktriangleup}), BG and MX plus MMS. Results are the means of at least three separate experiments performed in duplicate. *Bars*, SD.

Inhibitors of PARP Modulate the Sensitivity of Cells to TMZ.

Because inhibitors of PARP may interrupt BER and increase sensitivity to methylating agents, we examined whether inhibitors of PARP sensitize cells to TMZ. Figs. 3□ and 4□ display survival after combined treatment of TMZ with PD128763, 3-AB, or 6-AN in both SW480 and HCT116 cells. In the SW480 cell line, 100 μM PD128763 (IC_{50} for PD128763 alone, 625 μM) sensitized cells to TMZ with a DMF of 3.1 ± 0.12 ($P < 0.0002$). The combination of PD128763, BG, and TMZ was even more toxic, with a DMF of 36 (Fig. 3*A*)□. In HCT116 cells, the DMF for PD128763 and TMZ compared with TMZ alone was 4.7 ± 0.2 ($P < 0.0001$). However, the combination of PD128763, BG, and TMZ had no greater effect than PD128763 and TMZ (Fig. 4*A*)□, indicating that persistent O^6mG had no effect on cytotoxicity in this MMR-defective cell line. Potentiation of TMZ cytotoxicity was also observed in both cell lines treated with two other PARP inhibitors, 3-AB (Figs. 3*B*□ and 4*B*)□ and 6-AN (Figs. 3*C*□ and 4*C*)□. Although the specific activity of these agents varied considerably, DMF values of 3 to 4 were observed for both 3-AB and 6-AN when combined with TMZ compared to TMZ alone.

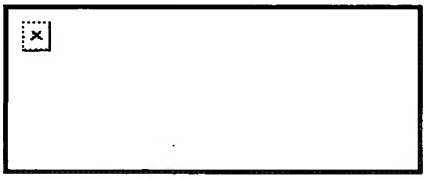

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Fig. 3. Inhibitors of PARP enhance cytotoxicity of TMZ in MMR wild-type SW480 cells. *A*, cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 100 μM PD128763 and/or 10 μM BG for 2 h prior to 2-h exposure to TMZ. ({blacksquare}), TMZ alone; (•), PD128763 plus TMZ; ({diamondsuit}), BG plus TMZ; ({blacktriangleup}), BG and PD128763 plus TMZ. *B*, cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 10 μM 6-AN (pretreated for 48 h) and/or 10 μM BG for 2 h

prior to 2-h exposure to TMZ. ({blacksquare}), TMZ alone; (•), 6-AN plus TMZ; ({diamondsuit}), BG plus TMZ; ({blacktriangleup}), BG and 6-AN plus TMZ. *C*, cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 3 mM 3-AB (pretreated for 24 h) and/or 10 μM BG for 2 h prior to 2-h exposure to TMZ. ({blacksquare}), TMZ alone; (•), 3-AB plus TMZ; ({diamondsuit}), BG plus TMZ; ({blacktriangleup}), BG and 3-AB plus TMZ. IC_{50} was 50 μM for 6-AN and >> 6 mM for 3-AB in this cells. Results are the means of at least three separate experiments performed in duplicate. *Bars*, SD.

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Fig. 4. Inhibitors of PARP enhance cytotoxicity of TMZ in MMR-deficient HCT116 cells. *A*, cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 100 μM PD128763 and/or 10 μM BG for 2 h prior to 2-h exposure to TMZ. ({blacksquare}), TMZ alone; (•), PD128763 plus TMZ; ({diamondsuit}), BG plus TMZ; ({blacktriangleup}), BG and PD128763 plus TMZ. *B*, cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 100 μM 6-AN (pretreated for 48 h) and/or 10 μM BG for 2 h prior to 2-h exposure to TMZ. ({blacksquare}), TMZ alone; (•), 6-AN plus TMZ; ({diamondsuit}), BG plus TMZ; ({blacktriangleup}), BG and 6-AN plus TMZ. *C*, cells were treated with 0–1500 μM TMZ for 2 h or TMZ plus 3 mM 3-AB (pretreated for 24 h) and/or 10 μM BG for 2 h prior to 2-h exposure to TMZ. ({blacksquare}), TMZ alone; (•), 3-AB plus TMZ; ({diamondsuit}), BG plus TMZ; ({blacktriangleup}), BG and 3-AB plus TMZ.

IC_{50} was 350 μM for 6-AN and >> 6 mM for 3-AB in this cells. Results are the means of at least three separate experiments performed in duplicate. *Bars*, SD.

Synergistic Interaction between TMZ and MX or PD128763.

We investigated the nature of the interaction between TMZ and MX in these two cell lines. These cells were incubated in the presence of a range of concentrations of TMZ (37.5–750 μM) and MX (0.75–15.0 mM) and a constant molar ratio mixture of TMZ and MX (1: 20), based on the relative IC_{50} for 2 h. Cells were also exposed to TMZ (18.8–750 μM) and PD128763 (15.6–625 μM) alone and at the fixed dose ratio of the combination of (1:0.83) for 2 h to analyze synergism. As shown in Fig. 5, synergistic interaction (C I<< 1; $P < 0.001$) was found in both SW480 and HCT116 cells for the combination of TMZ with either MX or PD128763 despite the fact that the HCT116 cells were TMZ resistant. This synergistic interaction was observed even at very low concentrations, which were absolutely nontoxic when each drug was used alone, indicating that BER inhibitors significantly synergize methylating agent cytotoxicity in both MMR-deficient and -proficient colon cancer cells.

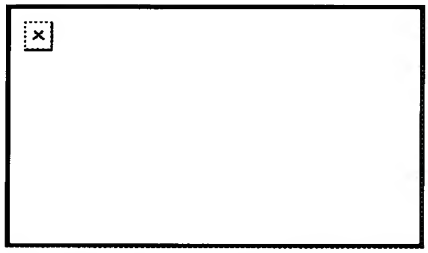

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Fig. 5. Synergy analysis of the interaction between TMZ and MX (*A*) or PD128763 (*B*) in SW480 ({blacktriangleup}), and HCT116 ({diamondsuit}) cells. Values are representative of two independent experiments.

Effect of BER Inhibitors on BCNU Cytotoxicity.

To test whether MX is also able to sensitize colon cancer cells to chloroethylating agents, these two cell lines were pretreated with 6 mM MX for 2 h, followed by BCNU. No enhancement of BCNU cytotoxicity by MX was observed (Fig. 6); the BCNU IC_{50} was 45 μM in HCT116 cells (Fig. 6*A*) and 27–29 μM, respectively, in SW480 cells treated with BCNU alone or BCNU plus MX (Fig. 6*B*). A greater sensitization to BCNU was observed in these two cell lines when cells were treated with MX plus BG and BCNU; the BCNU IC_{50} for both cell lines was 5 μM under these conditions. However, most of the effect was potentiation due to BG, which increased BCNU cytotoxicity by 3–4-fold. As shown in Fig. 7, no sensitization to BCNU cytotoxicity was seen after treatment with addition of PD 128763.

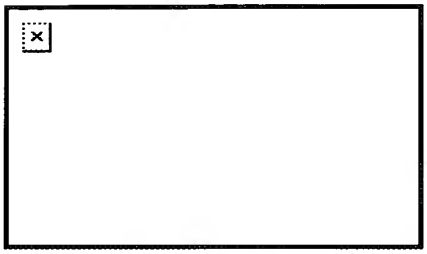

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Fig. 6. Survival fraction of human colon cancer cell lines after exposure to BCNU plus MX. Cells were treated with 0–100 μM BCNU for 2 h or BCNU plus 6 mM MX and/or 10 μM BG for 2 h prior to 2-h exposure to BCNU. *A*, HCT116 cells; *B*, SW480 cells. ({blacksquare}), BCNU alone; ({diamondsuit}), MX plus BCNU; (•), BG plus BCNU; ({blacktriangleup}), BG and MX plus BCNU. Results are the means of at least three separate experiments performed in duplicate. *Bars*, SD.

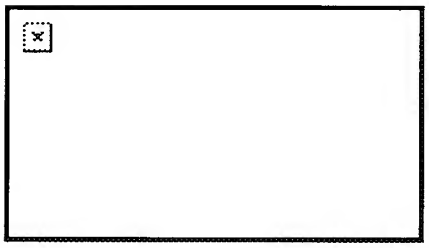

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Fig. 7. Survival fraction of human colon cancer cell lines after exposure to BCNU plus PD128763. Cells were treated0 with 0–100 μM BCNU for 2 h or BCNU plus 100 μM PD128763 and/or 10 μM BG for 2 h prior to 2-h exposure to BCNU. *A*, HCT116 cells; *B*, SW480 cells. ({blacksquare}), BCNU alone; ({diamondsuit}), PD128763 plus BCNU; (•), BG plus BCNU; ({blacktriangleup}), BG and PD128763 plus BCNU; ({blacktriangleright}) BG, PD128763 and MX plus BCNU. Results are the means of at least three separate experiments performed in duplicate. *Bars*, SD.

Effect of Inhibitors of BER on Cell Cycle Distribution and PARP Cleavage.
The cell cycle and apoptosis responses of SW480 and HCT116 cells were examined at various times after treatment with TMZ (300 μM) alone or with MX (6 mM), PD 128763 (100 μM), or BG (25 μM). After treatment, cells were divided into two aliquots for analysis of cell cycle/apoptosis on days 1 and 3, and for detection of PARP cleavage (see below). Cell cycle distribution was measured by flow cytometry according to DNA content, and estimation of the duration of the G_1, S, and G_2-M phases was based on untreated, exponentially growing, asynchronous cells. MX and PD128763 alone did not affect the distribution of cell cycle in these two cell lines (data not shown). At 24 h, 75–90% of SW480 cells accumulated in the S and G_2 phases after treatment with TMZ alone, and this S-G_2 phase arrest was more pronounced in cells pretreated with either MX or PD 128763 (Fig. 8*A*)□. S-G_2 phase arrest was still present after 3 days in cells treated with the combination of MX or PD 128763 and TMZ (in both instances, 13–20% of cells were

apoptotic). In SW480 cells treated with TMZ alone, the S-G_2 phase block was less obvious at day 3, with only 8% of cells showing evidence of apoptosis. In contrast, HCT116 cells had a normal cell cycle distribution after treatment with TMZ alone, and no effect was seen with BG and TMZ. However, accumulation in the S phase was observed (Fig. 8*B*)□ 24 h after treatment with PD 128763 plus TMZ. At 72 h, HCT116 cells had moved through the S phase, and thereafter, a significant portion of cells (90%) remained arrested in the G_2 phase with apoptosis present in 14% of cells. A similar but less striking result was observed with MX and TMZ in HCT116 cells. By 72 h, 60% of cells were still arrested in the S and G_2 phases and 10% of cells were apoptotic.

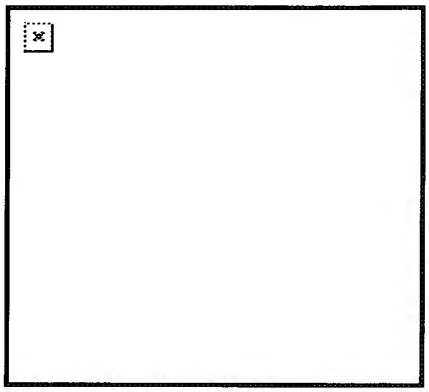

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Fig. 8. Distribution of cell cycle and apoptosis in colon cancer cell lines after treatment with TMZ plus modifiers. *A*, MMR wild-type SW480 cells; *B*, MMR-deficient HCT116 cells. *PD*, PD128763.

Finally, as a marker of apoptosis-induced cell death, we examined PARP cleavage after cells were treated with these drug combinations at 3 days (Fig. 9)□. PARP cleavage was observed in SW480 cells after exposure to TMZ alone and TMZ plus BG but was not seen in HCT116 cells with the same treatment, indicating that the apoptotic process is triggered when O^6mG lesions are repaired by the MMR system. However, PARP cleavage was detected in MMR-proficient and -deficient cells treated with TMZ plus either MX or PARP inhibitors.

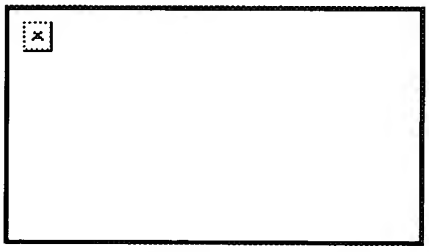

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Fig. 9. PARP cleavage in colon cancer cells treated with TMZ plus modifiers. *A*, SW480 and HCT116 cells untreated and treated with TMZ and BG; *B*, SW480 cells; *C*, HCT116 cells. *Lane 1*, untreated; *Lane 2*, TMZ + MX; *Lane 3*, TMZ + MX + BG; *Lane 4*, TMZ + 3-AB; *Lane 5*, TMZ + 3-AB + BG; *Lane 6*, TMZ + 6-AN; *Lane 7*, TMZ + 6-AN + BG; *Lane 8*, TMZ + PD128763; *Lane 9*, TMZ + PD128763 + BG. Representative blots from one of three

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DISCUSSION

Because MMR defective cell lines are remarkably resistant to methylating agents yet accumulate high levels of three methylating DNA adducts, O^6mG, N^3mA, and N^7mG, we reasoned that the interruption of repair of N^3mA and N^7mG adducts by the BER process would sensitize cells to methylating agents. To address this issue, we studied the effect of MX on potentiation of TMZ cytotoxicity. MX interacts specifically with the tautomeric open-ring form of deoxyribose generated from the removal of an abnormal base by glycosylase. The MX-modified AP site is relatively stable (26 , 27) and inhibits the cleavage of AP sites in DNA by AP endonuclease in mammalian cells. This has been shown to protect cells from cytotoxicity, mutagenicity induced by SN1-type ethylating agents, such as ethylnitrosourea, but not SN2 alkylating agents, such as diethyl sulfate and MMS (24 , 28) . Moreover, the protection was strictly time dependent and was limited to the short period (30 min) after exposure to the alkylating agents (28) . In our studies, we had also observed that MX reduced cleavage at AP sites and decreased BER in human colon cancer cell extracts.[5] However, we did not see protection of these two cell lines from ethylnitrosourea cytotoxicity when longer exposures to MX were used. The short duration of MX studied previously may not have the same impact on BER inhibition as does longer exposure to MX. Our results showed that MX synergistically increased TMZ-induced cytotoxicity in human colon cancer cell lines in both MMR-proficient and -deficient cells. A similar degree of enhanced cytotoxicity was observed with MX and MMS and with TMZ as well. The effect of BG inhibition of AGT was additive to the effect of MX only in the MMR-proficient SW480 cell line but not in the MMR-defective HCT116 cell line. These data suggest that O^6mG DNA adducts do not contribute to the enhanced cytotoxic effect of TMZ by MX. Furthermore, a similar degree of enhanced cytotoxicity was observed with MX and MMS as with TMZ, again implicating N^3mA- and N^7mG-induced abasic sites as the major targets for MX. In our recent studies, a prolonged exposure to low-dose MX results in even greater potentiation of TMZ cytotoxicity.

The mechanisms of MX-enhanced cytotoxicity of methylating agents in colon cancer cells have not been fully understood. It is fair to suggest that MX enhanced the cytotoxic effect of TMZ because (*a*) the MX-AP site complex is able to block the AP endonucleolytic step of the BER pathway; (*b*) the persistence of abasic sites may increase topoisomerase II-mediated DNA cleavage (29) ; and (*c*) AP sites inhibit DNA replication and trigger programmed cell death (30) .

Under normal circumstances, TMZ produces strand breaks during BER-mediated repair of N^7mG and N^3mA adducts that are repaired efficiently and do not contribute to cytotoxicity

until high concentrations of adducts are achieved. When DNA strand breaks are present, one component of the response is recognition, binding, and activation of PARP. Activated PARP leads to autoribosylation, and this in turn facilitates access of repair enzymes to DNA damage (15 , 31) and appears to enhance processing of stand breaks and religation by polymerase-{beta} and ligase I (32) . In the alternative BER pathway, PARP interacts with XRCC1 to facilitate repair (17) . It seems likely that PARP plays an important role in communication between repair proteins and the stability of the repair complex involved in BER. This suggests that inhibition of PARP leads to an impaired ability to rejoin DNA strand breaks, which can initiate both apoptotic and nonapoptotic cell death cascades and thereby increase cytotoxicity of TMZ (33) . Our results support this hypothesis. Potentiation of cytotoxicity of methylating agent with PARP inhibitors was observed with a marked increase in apoptosis and PARP cleavage.

The results of BCNU combined with either MX or PD128763 are in sharp contrast to TMZ: little if any potentiation is observed in the absence of BG in either cell line. This suggests that although BER appears to process BCNU-induced cross-links, inhibition of BER in this manner has little impact on BCNU toxicity. One of the best studied BCNU-induced lethal lesions is the N^3-cytosine-N^1-guanine interstrand cross-link formed after initial chloroethyl monoadducts at O^6-guanine and cyclic rearrangement to N^1,O^6-ethanoguanine (34) . However, treatment of cells with BCNU also produces alkylated bases that may be labile and spontaneously result in breakage or nicking of the phosphoribosyl backbone (35) . Because PARP has been shown to bind to BCNU-induced DNA nicks *in vitro* (36) , it is reasonable to assume that PD128763 might increase BCNU cytotoxicity. However, our studies showed only minor enhancement of toxicity in HCT116 cells and no enhancement in SW480 cells. Although methyladenine DNA glycosylase has been implicated in BCNU cross-link repair and its absence sensitizes cells to BCNU (37) , we did not observe sensitization to BCNU by treatment with MX in the absence of BG. In the presence of BG; MX potentiated BCNU toxicity, indicating that MX may interfere with DNA cross-link repair pathway and suggesting that BER may be involved in repair of the N^1,O^6-ethanoguanine cross-link, which is not formed if AGT reacts with the O^6-chloroethylguanine adduct. Taken together, these data suggest a different reaction of MX with damaged DNA induced by BCNU compared with TMZ. With TMZ, MX-increased cytotoxicity is associated with AP sites generated from repair of N^7mG and N^3mA DNA adducts formed by methylating agent; however; with BCNU, it might be the O^6 lesion-induced cross-link that controls BCNU toxicity.

It appeared that apoptosis mediates both MX and PD128763-enhanced cytotoxicity of TMZ. Increased apoptosis was observed in MMR wild-type SW480 cells but not in MMR-deficient HCT116 cells after treatment with BG and TMZ. This suggests that MMR processing of O^6mG is a potent apoptosis-inducing event (38) . Although the biological and functional consequences of PARP and its cleavage in apoptosis still remain to be further identified, it has been demonstrated that PARP is rapidly and specifically cleaved during apoptosis (39 , 40) . PARP cleavage was observed in both SW480 and HCT116

cells after treatment with either MX or one of the PARP inhibitors and TMZ, confirming activation of apoptotic pathways.

We noted that arrest at cell cycle checkpoints paralleled the cellular response to DNA damage and that these were dependent on MMR and BER pathways. MMR wild-type SW480 cells were sensitive to TMZ alone with arrest in the S and G_2 phases (2) . The S and G_2 phase arrests were potentiated by MX or by PD128763 despite the fact that SW480 is a p53 mutant cell line. In contrast, even high levels of DNA adducts formed by TMZ in the MMR-deficient HCT116 cells did not induce cell cycle checkpoint arrest despite the fact that p53 is wild type in this cell line. This dysregulation of damage-induced cell cycle checkpoint control appeared because of failure of processing O^6mG lesions in MMR-deficient cells. However, after combined treatment with TMZ and either MX or PD128763, HCT116 cells showed S-G_2 phase arrest and apoptosis. These results are consistent with previous studies of cell cycle changes after MMS exposure or other compounds that produce 90% N^3mA (41, 42, 43) and the prolonged G_2 phase arrest observed in PARP knockout mice or derived cell lines (22) following DNA damage. These data indicate that both SW480 and HCT116 cells have a similar response to persistent N^7mG and N^3mA lesions, following interruption of BER.

In summary, we have shown that disrupted BER processing of non-O^6mG, most likely N^7mG and N^3mA, DNA adducts formed by TMZ is cytotoxic to colon cancer cell lines. This may be particularly important in MMR-deficient cells, which are resistant to TMZ alone because of the failure to recognize O^6mG DNA adducts. These studies provide evidence that disrupting repair of N^7mG and N^3mA by inhibiting BER or PARP may improve the therapeutic efficacy of methylating agents.

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FOOTNOTES

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[3] The abbreviations used are: O^6mG, O^6-methylguanine; AGT, alkyltransferase; BG, O^6-benzylguanine; MMR, mismatch repair; TMZ, temozolomide; N^7mG, N^7-methylguanine; N^3mA, N^3-methyladenine; BER, base excision repair; AP, apurinic-apyrimidinic; PARP, poly(ADP-ribose) polymerase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; MX, methoxyamine; PD128763, 3,4-dihydro-5-methyoxyisoquinoline-1(2*H*)-one; 3-AB, 3-aminobenzamide; 6-AN, 6-aminonicotinamide; MMS, methylmethane sulfonate; DMF, dose modification factor. ☐

[4] L. Liu and S. L. Gerson, unpublished results. ☐

[5] P. Taverna, L. Liu, and S. L. Gerson, unpublished data. ☐

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☐ REFERENCES

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1. Pegg A. E., Dolan M. E., Moschel R. C. Structure, function and inhibition of O^6-alkylguanine-DNA AGT. Prog. Nucleic Acid Res. Mol. Biol., *51:* 167-233, 1995.[Medline]
2. Fink D., Aebi S., Howell S. B. The role of DNA mismatch repair in drug resistance. Clin. Cancer Res., *4:* 1-6, 1998.[Abstract]
3. Branch P., Aquilina G., Bignami M., Karran P. Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. Nature (Lond.), *362:* 652-654, 1993.[Medline]
4. Kat A., Thilly W. C., Fang W-H., Longley M. J., Li G-M., Modrich P. An alkylation-tolerant, mutator human cell line is deficient in strand-specific mismatch repair. Proc. Natl. Acad. Sci. USA, *90:* 6424-6428, 1993.[Abstract]
5. Karran P., Bignami M. DNA damage tolerance, mismatch repair and genome instability. BioEssays, *16:* 833-839, 1994.[Medline]
6. Griffin S., Branch P., Xu Y-Z., Karran P. DNA mismatch binding and incision at modified guanine bases by extracts of mammalian cells: implications for tolerance to DNA methylation damage. Biochemistry, *33:* 4787-4793, 1994.[Medline]
7. Liu L., Markowitz S., Gerson S. L. Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide by not to 1, 3-bis(2-chloroethyl)nitrosourea. Cancer Res., *56:* 5375-5379, 1996.[Abstract]
8. O'Connor T. R., Laval F. Isolation and structure of a cDNA expressing a mammalian 3-methyladenine-DNA glycosylase. EMBO J., *9:* 3337-3342, 1990. [Abstract]
9. Samson L., Derfkerm B., Boosalis M., Call K. Cloning and characterization of a 3-methyladenine DNA glycosylase cDNA from human cells whose gene maps to chromosome 16. Proc. Natl. Acad. Sci. USA, *88:* 9127-9131, 1991.[Abstract]

10. Neddermann P., Gallinari P., Lettieri T., Schmid D., Truong O., Hsuan J. J., Wiebauer K., Jiricny J. Functional expression of soluble human interleukin-11 (IL-11) receptor {alpha} and stoichiometry of in vitro IL-11 receptor complexes with gp130. J. Biol. Chem., *271:* 12767-12774, 1996.[Abstract/Free Full Text]
11. Vollberg T. M., Siegler K. M., Cool B. L., Sirover M. A. Isolation and characterization of the human uracil DNA glycosylase gene. Proc. Natl. Acad. Sci. USA, *86:* 8693-8697, 1989.[Medline]
12. Olsen L. C., Aasland R., Wittwer C. U., Krokan H. E., Helland D. E. Molecular cloning of human uracil-DNA glycosylase, a highly conserved DNA repair enzyme. EMBO J., *8:* 3121-3125, 1989.[Abstract]
13. Radicella J. P., Dherin C., Desmaze C., Fox M. S., Boiteux S. Cloning and characterization of *hOGG1*, a human homolog of the *OGG1* gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA, *94:* 8010-8015, 1997. [Abstract/Free Full Text]
14. Rosenquist T. A., Zharkov D. O., Grollman A. P. Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. Proc. Natl. Acad. Sci. USA, *94:* 7429-7434, 1997.[Abstract/Free Full Text]
15. Willson S. H. Mammalian base excision repair and DNA polymerase {beta}. Mutation Res., *407:* 203-215, 1998.[Medline]
16. Molinete M., Vermeulen W., Burkle A., Menissier-de Murcia J., Kupper J. H., Hoeijmakers J. H. J., de Murcia G. Overproduction of the poly (ADP-ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mammalian cells. EMBO J., *12:* 2109-2117, 1993.[Abstract]
17. Caldecott K. W., Aoufouchi S., Johnson P., Shall S. XRCC1 polypeptide interacts with DNA polymerase {beta} and possibly poly(ADP-ribose) polymerase, and DNA ligase III is a novel molecular 'nick-sensor' in vitro. Nucleic Acids Res., *24:* 4387-4394, 1996.[Abstract/Free Full Text]
18. Sobol R. W., Horton J. K., Kuhn R., Gu H., Singhal R. K., Prasad R., Rajewsky K., Wilson S. H. Requirement of mammalian DNA polymerase-{beta} in base-excision repair. Nature (Lond.), *379:* 183-186, 1996.[Medline]
19. Walker L. J., Craig R. B., Harris A. L., Hickson I. D. A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress. Nucleic Acids Res., *22:* 4884-4889, 1994.[Abstract]
20. Engelward B. P., Dreslin A., Christensen J., Huszar D., Kurahara C., Samson L. Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. EMBO J., *15:* 945-952, 1996.[Abstract]
21. Coquerelle T., Dosch J., Kaina B. Overexpression of *N*-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents—a case of imbalanced DNA repair. Mutat. Res., *336:* 9-17, 1995.[Medline]
22. Menissier-de Murcia J., Niedergang C., Trucco C., Ricoul M., Dutrillaux B., Mark M., Oliver F. J., Masson M., Dierich A., LeMeur M., Walztinger C., Chambon P., de Murcia G. Requirement of poly(ADP-ribose) polymerase in recovery from DNA damage in mice and in cells. Proc. Natl. Acad. Sci. USA, *94:* 7303-7304, 1997. [Abstract/Free Full Text]
23. Boulton S., Pemberton L. C., Porteous J. K., Curtin N. J., Griffin R. J., Golding B. T., Durkacz B. W. Potentiation of temozolomide-induced cytotoxicity: a comparative study of the biological effects of poly(ADP-ribose) polymerase

inhibitors. Br. J. Cancer, *72:* 849-856, 1995.[Medline]
24. Fortini P., Calcagnile A., Vrieling H., van Zeeland A. A., Bignami M., Dogliotti E. Mutagenic processing of ethylation damage in mammalian cells: the use of methoxyamine to study apurinic/apyrimidinic site-induced mutagenesis. Cancer Res., *53:* 1149-1155, 1993.[Abstract]
25. Chou T. C., Talalay P. Quantitative analysis of dose-effect relationship: the combined effects of multiple drugs on enzyme inhibitors Weber G. eds. . Advances in Enzyme Regulation, *:* 27-55, Pergamon Press New York 1983.
26. Liuzzi M., Talpeart-Borle M. A new approach to the study of the base-excision repair pathway using methoxyamine. J. Biol. Chem., *260:* 5252-5258, 1985. [Abstract]
27. Rosa S., Fortini P., Bignami M., Dogliotti E. Processing in vitro of an abasic site reacted with methoxyamine: a new assay for the detection of abasic sites formed in vivo. Nucleic Acids Res., *19:* 5569-5574, 1991.[Abstract]
28. Fortini P., Rosa S., Zijno A., Calcagnile A., Bignami M., Dogliotti E. Methoxyamine modification of abasic sites protects CHO cells from the cytotoxic and mutagenic effects of oxygen alkylation. Carcinogenesis (Lond.), *13:* 87-93, 1992.[Abstract]
29. Kingma P. S., Osheroff N. Apurinic sites are position-specific topoisomerase II poisons. J. Biol. Chem., *272:* 1148-1155, 1997.[Abstract/Free Full Text]
30. Robertson K. A., Hill D. P., Xu Y., Liu L., Epps S. V., Hockenbery D. M., Park J. R., Wilson T. M., Kalley M. R. Down-regulation of apurinic/apyrimidinic endonuclease expression is associated with the induction of apoptosis in differentiating myeloid leukemia cells. Cell Growth & Differ., *8:* 443-449, 1997. [Abstract]
31. Buschfort C., Muller M. R., Seeber S., Rajewsky M. F., Thomale J. DNA excision repair profiles of normal and leukemic human lymphocytes: functional analysis at the single-cell level. Cancer Res., *57:* 651-658, 1997.[Abstract]
32. Althaus F. R., Hofferer L., Kleczkowska H. E., Malanga M., Naegeli H., Panzeter P., Realini C. Histone shuttledriven by the automodification cycle of poly(ADP-ribose) polymerase. Environ. Mol. Mutagen., *22:* 278-282, 1993.[Medline]
33. Wedge S. R., Porteous J. K., Newlands E. S. 3-Aminobenzamide and/or O^6-benzylguanine evaluated as an adjuvant to temozolomide or BCNU treatment in cell lines of variable mismatch repair status and O^6-alkylguanine-DNA alkyltransferase activity. Br. J. Cancer, *74:* 1030-1036, 1996.[Medline]
34. Wilson D. M., III, Thompson L. H. Life without DNA repair. Proc. Natl. Acad. Sci. USA, *94:* 7303-7304, 1997.[Abstract/Free Full Text]
35. Gonzaga P. E., Potter P. M., Niu T. Q., Yu D., Ludlum D. B., Rafferty J. A. Identification of the cross-link between human O^6-methylguanine-DNA methyltransferase and chloroethylnitrosourea-treated DNA. Cancer Res., *52:* 6052-6058, 1992.[Abstract]
36. Prakash A. S., Gibson N. W. Sequence-selective depurination, DNA interstrand cross-linking and DNA strand break formation associated with alkylated DNA. Carcinogenesis (Lond.)., *13:* 425-431, 1992.[Abstract]
37. Malapetsa A., Noe A. J., Poirier G. G., Desnoyers S., Berger N. A., Panasci L. C. Identification of a 116 kDa protein able to bind 1, 3-bis (2-chloroethyl)-1-nitrosourea-damaged DNA as poly(ADP-ribose) polymerase. Mutat. Res., *362:* 41-50, 1996.[Medline]
38. Matijasevic Z., Boosalis M., Mackay W., Samson L., Ludlum D. B. Protection against chloroethylnitrosourea cytotoxicity by eukaryotic 3-methyladenine DNA glycosylase. Proc. Natl. Acad. Sci. USA, *90:* 11855-11859, 1993.[Abstract]
39. Kaina B., Ziouta A., Ochs K., Coquerelle T. Chromosomal instability, reproductive

cell death and apoptosis induced by O^6-methylguanine in Mex-, Mex+ and methylation-tolerant mismatch repair compromised cells: facts and models. Mutat. Res., *381:* 227-241, 1997.[Medline]

40. Lindahl T., Satoh M. S., Poirier G. G., Klungland A. Post-translational modification of poly(ADP-ribose) polymerase induced by DNA strand breaks. Trends Biochem. Sci., *20:* 405-411, 1995.[Medline]
41. Lazebnik Y. A., Kaufmann S. H., Desnoyers S., Poirier G. G., Earnshaw W. C. Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature (Lond.), *371:* 346-347, 1994.[Medline]
42. Morris S. M., Domon O. E., McGarrity L. J., Kodell R. L., Casciano D. A. Flow cytometric evaluation of cell-cycle progression in ethylmethanesulfonate and methylmethonesulfonate exposed P3 cells: relationship to the induction of sister-chromatid exchanges and cellular toxicity. Environ. Mol. Mutagen., *18:* 139-149, 1991.[Medline]
43. Engelward B. P., Allan J. M., Dreslin A. J., Kelly J. D., Wu M. M., Gold B., Samson L. D. A chemical and genetic approach together define the biological consequences of 3-methyladenine lesion in the mammalian genome. J. Biol. Chem., *273:* 5412-5418, 1998.[Abstract/Free Full Text]

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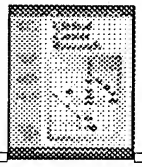

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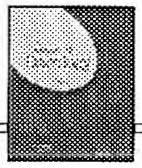

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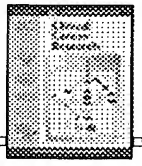

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